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Title

Detection of Cry9C protein in dry milled, wet milled and masa processed fractions and processed foods made from 100% StarLink™ grain

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Completed On

17 April 2001

Sponsor

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STATEMENT OF **NO** CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA 10(d) (1) (A). (B). (C).

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Aventis CropScience USA LP

Date: 17 Apr 01

Good Laboratory Practice Compliance Statement

The undersigned hereby declare that the work to which this report refers was performed according to the procedures herein described and this report provides an accurate record of the results obtained. The study was conducted in accordance with the Good Laboratory Practice Standards as specified in 40 CFR 160 except as follows:

General:

1. Amendments 4, 5 and 6 were not issued in a timely fashion [40 CFR 160.33(a)].

Source of the grain:

1. The StarLink and non-StarLink grain was obtained from farmers' fields. Weather and crop records were therefore not obtained under GLPS. [40 CFR 160.1].
2. The Certificate of Analysis was not produced under GLPS. [40 CFR 160.1].

Food sample preparation:

1. Food preparation at Texas A&M University, College Station, TX, F.R.I. Enterprises, New Berlin, WI, Diehl, Inc., Defiance, OH, and The National Food Laboratory, Dublin, CA was not carried out under GLPS, and therefore any reports from these laboratories are not included in this report However, the methods used are described in appendix 1 on the basis of communications with the processors. [40 CFR 160.1].

Analytical:

1. An equipment log was lacking for the plate reader; therefore, records are lacking of inspection, maintenance, calibration, and standardization per 40 CFR 160.63 (c).
2. SOPs were available for equipment but they lacked some of the elements required in 40 CFR 160.63(b).
3. Training files were available but they lacked some of the elements required in 40 CFR 160.29(b).
4. An SOP was lacking for data handling, storage, and retrieval per 40 CFR 160.81 (b).
5. Data are lacking for the synthesis, characterization, and stability of the Cry9C and antibodies. [40 CFR 160.105 and 160.185 (a) (5)].
6. Raw data on the validation of the EnviroLogix assay for Baked Taco shells is misplaced [40 CFR 160.190(a)].

Good Laboratory Practice Compliance Statement (continued)

Study Director

William J. Kowite

William J. Kowite, Ph.D.
Senior Research Scientist, Biotechnology Support

April 17, 2001
Date

Sponsor

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April 17, 2001
Date

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Luann Powell

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17 Apr. 01
Date

QUALITY ASSURANCE STATEMENT

This study was inspected and the findings reported to the facility management and to the study director on the listed dates:

Inspection Concluded	QAU of	Date Reported to Study Director	Date Reported to Facility Management
10/12/2000	Aventis CropScience	10/12/2000	10/12/2000
10/16/2000	Land O'Lakes Research Farm	11/17/2000	3/27/2001
1/3/2001	Food Protein Research and Development, TexasA&M	1/24/2001	1/24/2001
1/8/2001	Food Protein Research and Development, TexasA&M	1/24/2001	1/24/2001
1/12/2001	Aventis CropScience	1/29/2001	1/29/2001
1/22/2001	Aventis CropScience	3/1/2001	3/1/2001
3/29/2001	Aventis CropScience	4/5/2001	4/5/2001
3/30/2001	Food Protein Research and Development, TexasA&M	4/2/2001	4/2/2001
3/30/2001	Food Protein Research and Development, TexasA&M	4/2/2001	4/2/2001
4/5/2001	Aventis CropScience	4/5/2001	4/5/2001
4/8/2001	Aventis CropScience	4/8/2001	4/8/2001

Quality Assurance Unit George R Butler April 17, 2001
Date

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: **Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods**

In compliance with the Good Laboratory Practice regulations an inspector with the Quality Assurance Unit has inspected at least one phase of this study. Inspection findings were reported to GLP Program management, the study director and the study director's management. The Quality Assurance Unit has reviewed the processing report and certifies that it accurately describes the methods and standard operating procedures used, and the reported results accurately reflect the raw data generated during this processing phase.

Signed: _____ Date: 02 Apr 2001

Doyle L. Borchgardt
Quality Assurance Coordinator
Food Protein Research and Development Center

INSPECTION		DATES REPORTED TO:	
TYPE	DATE	GLP PROGRAM MANAGEMENT	STUDY DIRECTOR & STUDY DIRECTOR'S MANAGEMENT
1) Process Phase SOP 8.6 R11, Section 4: "Solvent Extraction of Germ Oil"	02 & 03 Jan 2001	09 Jan 2001	24 Jan 2001
2) Process Report	27 thru 30 Mar 2001	30 Mar 2001	02 Apr 2001

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: **Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods**

In compliance with the Good Laboratory Practice regulations an inspector with the Quality Assurance Unit has inspected at least one phase of this study. Inspection findings were reported to GLP Program management, the study director and the study director's management. The Quality Assurance Unit has reviewed the processing report and certifies that it accurately describes the methods and standard operating procedures used, and the reported results accurately reflect the raw data generated during this processing phase.

Signed: _____ Date: 02 Apr 2001

Doyle L. Borchgardt
Quality Assurance Coordinator
Food Protein Research and Development Center

INSPECTION		DATES REPORTED TO:	
TYPE	DATE	GLP PROGRAM MANAGEMENT	STUDY DIRECTOR & STUDY DIRECTOR'S MANAGEMENT
1) Process Phase SOP 8.13 R. 07: "Laboratory Deodorization of Vegetable Oil"	08 Jan 01	09 Jan 2001	24 Jan 2001
2) Process Report	27 thru 30 Mar 2001	30 Mar 2001	02 Apr 2001

APPROVALS PAGE

Study Director/ Author: William J. Kowite — April 17, 2001

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Senior Research Scientist, Biotechnology Support

Date

Author: Susan MacIntosh — Apr 17, 2001

Susan MacIntosh
Global Product Safety Management, Regulatory Affairs

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Author/Sponsor: Raymond D Shillito — April 17, 2001

Raymond D. Shillito, Ph.D.
Manager, Biotechnology Support

Date

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SUMMARY

This study was undertaken for two purposes. The main purpose of this study was to determine the effects of processing of wet and dry milled fractions and masa processed fractions and other foods made from 100% StarLink corn on detectable levels of Cry9C protein. The grain used for this study consisted of grain harvested from a field planted to 100% StarLink™ hybrid corn. Food products that had a wide range of different processing and cooking conditions were prepared from this grain. Some foods were prepared at more than one facility and, in some cases, more than one batch of a food was prepared in order to ensure that representative samples from typical processing methods and recipes were prepared. In all cases, the processing of fractions and the food preparation were done to mimic real commercial practices as much as possible. Small-scale processors make every effort to mimic commercial scale production, but generally the commercial processes would be harsher on proteins than the small-scale processing methods.

The second objective was to compare the two different ELISA (Enzyme Linked ImmunoSorbent Assay) methods, and determine which ELISA method is the more sensitive for detecting the presence or absence of the Cry9C protein in food ingredients or finished products. The processed food products were tested using ELISA methods, which is the generally accepted standard method for quantifying proteins. A range of wet milled, dry milled and processed food products were analyzed using two different ELISA methods – the EnviroLogix and the Aventis ELISA methods – each of which is based on polyclonal antibodies. It is not surprising to see differences from one ELISA method to another, especially since the extraction buffers are different.

Although ELISA methods are very sensitive they are also expensive, time consuming and more difficult to perform than another immunologically based method, the lateral flow strip test (Strategic Diagnostics, Inc. and EnviroLogix, Inc.). An ELISA test is generally performed in a well equipped laboratory setting by trained laboratory personnel. Strip tests, on the other had, are quick and easy to perform and can detect one StarLink™ kernel in a sample of 800 kernels or about 20 ppb of Cry9C protein. Strip tests are simple to perform by personnel with minimal training in almost any environment: field, lab, processing facility and grain elevators. The two ELISA methods produced very similar quantitative values for each matrix tested, however, the amounts of Cry9C protein detected in the various finished corn products were in general higher when using the Envirologix method.

This study reveals the impact of processing on the fate of the Cry9C protein in finished foods. The results demonstrate that there is extensive reduction in the amount of detectable Cry9C protein during processing of 100% StarLink™ grain into processed corn food products. All processing methods reduce the amount of Cry9C protein significantly. The loss of Cry9C protein is due to a combination of recipe dilution, processing methods and cooking. The degree of the reduction depends on the specific processing method used. Three factors appear to cause destruction of the Cry9C protein: heat, shear or pressure, and alkali treatment. The greater the dilution and the more harsh the processing/cooking (heat, shear or pressure and alkali treatment), the lower the level of the Cry9C protein in the finished food product.

The Cry9C protein levels detected in these finished foods represent a worst case scenario, for two reasons. First, the foods in question were made from 100% StarLink grain. Foods made from 100% StarLink™ grain are not available to consumers in the marketplace. Second, the foods tested were

produced in small scale processing rather than by commercial processing and therefore are likely to overstate the amount of Cry9C that would remain after harsher commercial processing methods.

The quantities of the Cry9C protein found in the wet and dry milled fractions were comparable to the quantities found in earlier processing studies.

The wet milled production of starch from 100% StarLink™ grain led to the loss of more than 99.9% of the Cry9C protein and no Cry9C was detected in the refined oil. The Cry9C protein level in high fructose corn syrups is likely to be reduced even more because syrup products are processed from wet milled starch. These findings are consistent with the calculated values given by the EPA in their White Paper on Wet Milling (EPA, 2001).

Foods produced by the Masa process were also extremely low in Cry9C protein. Cry9C protein was detected, at 23.6 and 20.3 ppb, in only two of the six different samples tested. The amount of Cry9C protein was below the detection limit in the remaining samples.

Seven samples which had been collected from grocery store shelves in September 2000 during a recall of taco shells, and which had tested positive for *cry9C* DNA were also assayed. Very low levels of the Cry9C protein (1 to 4 ppb) were detected in four of the six PCR-positive taco shell products, and one sample contained a trace amount (<LOQ) of the Cry9C protein. The other two samples were below the detection limit of the ELISA assay. These Cry9C protein levels would not be found in commercial tortilla products produced today, due to the grain testing program which has been put in place at the grain elevators and mills by the USDA Grain Inspection, Packers and Stockyard Administration (GIPSA-FGIS directive 9181.1, and bulletin #191). This program utilizes lateral flow strips, as described above, with a sensitivity level of about 20 ppb and directs corn that tests positive to permitted animal feed and industrial uses.

Less than 5 ppb of the Cry9C protein was found in corn snacks and cereal corn products produced using the degermed corn meal fraction of 100% StarLink™ grain. The highest Cry9C protein levels, ranging from approximately 450 to 2700 ppb, were detected in polenta, corn bread, corn muffins and hush puppies. Although these levels are based on cooked products, the Cry9C protein levels in uncooked mixes for corn bread, corn muffins, hush puppies and polenta would be higher, and perhaps as much as three times the values determined from the cooked products.

In conclusion, the data from all products consistently indicate that food processing causes a dramatic reduction in Cry9C protein levels in the finished product when compared to the raw commodity from which the finished product is made. Although the change in the absolute values reflected in this study demonstrates the impact of processing on Cry9C protein levels, the resulting Cry9C protein levels are overstated because they are based on a starting point of 100% StarLink corn. The current grain testing program is designed to prevent corn with more than 20 ppb Cry9C protein from entering the human food supply.

STUDY IDENTIFICATION

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Study Initiation Date: October 20, 2000
Experimental Start Date: November 8, 2000
Experimental Termination Date: March 29, 2001

ACRONYMS and. SCIENTIFIC TERMS

BTID – Biotechnology Support Identification number
BSA – Bovine Serum Albumin
CV – Coefficient of Variation
ELISA – Enzyme Linked ImmunoSorbent Assay
GIPSA – Grain Inspection, Packers and Stockyard Administration
GLP – Good Laboratory Practice
LOD – Limit of Detection
LOQ – Limit of Quantitation
NA – Not Applicable
ND – Not Detectable: Below the limit of detection
NT – Non-StarLink
OD – Optical Density
PCR – Polymerase chain reaction
ppb – Parts per billion
SD – Standard Deviation
SEB – standard extraction buffer
TEP – Total Extractable Protein
USA – United States of America
USDA – United States Department of Agriculture

1. INTRODUCTION

Information on the analysis of wet milled and dry milled products from StarLink™ field grain was reported previously (Shillito, 1998). The ELISA (Enzyme Linked ImmunoSorbent Assay) is the generally accepted standard method for quantifying proteins. While ELISA methods are quite sensitive they are also more expensive, take more time and are more difficult to perform than another immunologically based method, the lateral flow strip test (Strategic Diagnostics, Inc. and EnviroLogix, Inc.). An ELISA test is generally performed in a well equipped laboratory setting by trained laboratory personnel. Strip tests, on the other had, are quick and easy to perform and can detect one StarLink kernel in a sample of 800 kernels or about 20 ppb of Cry9C protein. Strip tests are simple to perform in most any environment, field, lab, processing facility and grain elevators, by personnel with minimal training.

A previous study on the fate of Cry9C protein during masa processing (Shillito and Artis, 2000) used an ELISA method which employed a monoclonal antibody. Polyclonal antibodies are generally better suited for detecting denatured or partially denatured proteins, and provide more accurate results on protein levels in processed foods. The purpose of the current study was thus to apply and compare two different polyclonal antibody-based ELISA methods for the detection of Cry9C protein in corn products from wet and dry milling, and from masa processed and other processed foods. The same set of samples has also been analyzed using a quantitative DNA assay (Appendix 9). All of these corn products (fractions and processed foods) were prepared from grain harvested from a field planted to 100% StarLink™ corn hybrid (referred to here as 100% StarLink grain). The same products were also prepared from non-StarLink grain in order to allow for validation of the assay in the various matrices.

2. COMPOUND INFORMATION

Reference Substances

a) Cry9C protein
Chemical name: Insecticidal Crystal Protein 9C
Molecular Weight: 70 kDa

Cry9C protein reference substances and antibodies specifically recognizing each target protein were supplied by Aventis CropScience N.V., (Gent, Belgium). Upon arrival at Aventis CropScience USA (formerly AgrEvo USA Company), each component was assigned a unique lot number. Cry9C reference substance was also used to fortify non-StarLink samples for validation and recovery studies.

b) Bovine Serum Albumin
Chemical Name: Bovine Serum Albumin (BSA)
Molecular Weight: 66.4 KDa

BSA (Sigma, Product No: B6916) was used as standard for Bradford assay to measure the total extractable protein in the extracts (TEP: see Appendix 3).

3. PRODUCTION OF SAMPLES

3.1 Origin of Corn Grain

The StarLink grain (Garst 8600 BLT) was obtained by Land O'Lakes Research Farm in Iowa and was assigned the sample number CM00B010-03. The grain was harvested from a local grower's field and the presence of StarLink grain was confirmed using the Strategic Diagnostics, Inc., test kit (Trait√ Bt9 Corn Grain Test Kit, Part Number 7000034).

It is implicit in the way that the sample was obtained that not every corn kernel within this batch of 100% StarLink grain contains the Cry9C protein, since the Cry9C protein found in StarLink hybrid corn seed, event CBH351, is expressed as a hemizygous trait. Only half of the pollen and ovules from each StarLink corn plant contains the *cry9C* gene and the other half does not. When pollen from a StarLink corn plant pollinates ovules from the same plant, the resulting kernels will have two copies, one copy or no copies of the *cry9C* gene (at a 1:2:1 ratio respectively). Thus only 75% of the corn kernels actually contain the *cry9C* gene and therefore express the Cry9C protein. However, this material does accurately reflect the level of the Cry9C protein from grain harvested by a farmer from a 100% StarLink corn field.

The control field grain variety was Pioneer 3751, which was sampled by Qualls Agricultural Laboratory in Washington State from a grain bin located on a nearby farm. Corn containing *Bt* genes is not normally grown in this area, so pollen contamination from *Bt* genes is unlikely. Additionally, no StarLink corn seed was sold or planted in the state of Washington, nor was it sold or planted within the surrounding states adjacent to Washington State. The control grain was assigned the sample number CM00B010-04. This grain was tested using the Strategic Diagnostics Inc. test kit (Trait√ Bt9 Corn Grain Test Kit, Part Number 7000034). The grain tested negative for the presence of StarLink kernels with a 95% certainty of containing less than 0.19% StarLink. A number of samples of this grain have been tested by different laboratories and found to be free of the Cry9C protein expressed in StarLink grain (data not shown). A portion of the grain was shipped to the GLP Processing Program of Texas A&M University.

Control grain and StarLink grain were shipped to the GLP Processing Program of Texas A&M University, and F.R.I. Enterprises New Berlin, WI, as well as to Aventis CropScience, Research Triangle Park, NC.

3.2 Certificate of Analysis

Samples of raw corn grain, and of the masa and soft tortillas prepared at Texas A&M University were used to produce a Certificate of Analysis. This certificate (COA BTS0007/01) is included as Appendix 6. The analysis showed that the StarLink grain, along with masa and soft tortillas prepared from it, contained the *cry9C* DNA, and that the non-StarLink grain masa and soft tortillas did not contain *cry9C* DNA. In addition, every analysis carried out on this grain and the processed food samples has shown that the non-StarLink samples did not contain any Cry9C protein or *cry9C* DNA. The data show that the samples shipped to Texas A&M and Aventis CropScience originated from a sample of StarLink and non-StarLink grain, as expected.

3.3 Processing

Wet Milled Fractions

Wet milled fractions (Table 1) were produced from non-StarLink and 100% StarLink grain by the Food Protein Research laboratory, College Station, TX, by Dick Dusek as Principal Investigator under the supervision of Malcolm Gerngross. The wet milled commodities produced for analysis were starch, gluten, hulls, steepwater concentrate, solvent extracted germ, and refined oil. Details are given in Appendix 1.

Table 1: Sample List (Wet milled samples)

Product:	**Prepared by:**	**Assigned BTID[1]:**	
		Prepared from Control	**Prepared from StarLink**
Wet milled starch	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	454B	455B
Wet milled gluten	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	454C	455C
Wet milled hull	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	454D	455D
Steepwater Concentrate	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	454E	455E
Solvent extracted germ	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	457A	457C
Refined oil	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	457B	457D

[1]BTID = Biotechnology Support Identification number

Dry Milled Fractions

Dry milled fractions (Table 2) were prepared from non-StarLink and 100% StarLink grain. The 100% StarLink grain and the control Pioneer grain were degermed and processed into fine corn meal (approximately –30/+60 U.S. Standard Sieve size) and corn flour by the GLP Processing Program at the Food Protein Research laboratory, College Station, TX. A more complete description of the preparation of the dry milled samples is given in Appendix 1.

Table 2: Sample List (Dry milled samples)

Product:	**Prepared by:**	**Assigned BTID[1]:**	
		Prepared from Control	**Prepared from StarLink**
Whole grain	Not applicable	454A	455A
Corn meal	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	459A	459B
Corn flour	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	461A	461B

[1]BTID = Biotechnology Support Identification number

Processed Food samples

Processed food products (Table 3) were prepared directly from 100% StarLink grain and Pioneer 3751 (non-StarLink control) grain, or from fine corn meal produced during dry milling . A description of the preparation of the samples is given below and in more detail in Appendix 1.

Table 3: Sample List (Processed food samples)

Product:	**Prepared by:**	**Assigned BTID[1]:**	
		Prepared from Control	**Prepared from StarLink**
Masa (dough)	Texas A&M University College Station, TX (Dr. Lloyd Rooney)	418D	414C
Tortillas (soft)	Texas A&M University College Station, TX (Dr. Lloyd Rooney)	418N	414A
Tortilla chips (fried)	Texas A&M University College Station, TX (Dr. Lloyd Rooney)	418M	414B
Corn puffs	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	450B	450D
"Ringed" cereal (Cheerios-like)	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	450A	450C
Tortillas (soft)	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	451A	451B
Corn puffs	Diehl, Inc. Defiance, OH (Tom Diehl)	452A	452B

Product:	**Prepared by:**	**Assigned BTID[1]:**	
		Prepared from Control	**Prepared from StarLink**
Corn flakes	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	453A	453B
Tortilla chips (fried)	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	449A, B	449D, E
Taco shells (baked)	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	449C	449F
Polenta	The National Food Laboratory Dublin, CA (Debbie Lohmeyer)	456A, B	456C, D
Hush puppies	The National Food Laboratory Dublin, CA (Debbie Lohmeyer)	456E	456H
Corn muffins	The National Food Laboratory Dublin, CA (Debbie Lohmeyer)	456F, G	456I, J
Corn bread	The National Food Laboratory Dublin, CA (Debbie Lohmeyer)	456K, L	456M, N

[1]BTID = Biotechnology Support Identification number

Masa, Tortillas and Tortilla Chips

Masa (dough), soft tortillas and tortilla chips were produced by Dr. Lloyd W. Rooney, at the Cereal Quality Lab, College Station, TX. as reported previously in Aventis report CM00B011 (Aventis Document B003088, MRID 452753-01).

Prior to cooking, both the StarLink grain and the control non-StarLink grain was cleaned by aspiration and screening to remove impurities such as dust, chaff and weed seeds. The cleaned grain was placed in nylon mesh bags, which were suspended in near-boiling water containing pickling lime in a steam-jacketed stainless steel kettle. The quantity of lime used was approximately 1% of the grain weight in the batch. Continued steam heating brought the temperature back to a simmering boil (about 97°C). The grain was then cooked for seven minutes at a low boil, with stirring about once every minute. The steam was then turned off, and a lid was placed on the kettle. The grain was steeped (soaked) in the alkaline liquor overnight (about 15 hours).

The next morning, the alkali-cooked grain, or Nixtamal, was removed from the cooking kettle and washed with tap water in a bucket. The cooked kernels were hand-rubbed to remove most of the pericarp (hull material) and the washed Nixtamal was stone ground, thus being sheared

and mashed to produce fresh masa. From the stone grinder, the fresh masa was run through steel rollers and formed into sheets. The sheet of masa was cut into disks. The masa disks were cooked in a triple pass gas-fired oven, where they were baked into tortillas. The baking time in the oven was about one minute. The baking temperatures within the three-tiered oven were about 320°C (top), 280°C (middle) and 240°C (bottom).

The soft tortillas were cooled for about 10 minutes and cut into sections, which were cooked in commercial corn oil in a commercial-type deep fat fryer at approximately 190°C for 40 seconds to produce tortilla chips.

Masa, Tortillas, Taco Shells and Tortilla Chips

Tortillas, taco shells and tortilla chips were prepared by Dr. Triveni Shukla, F.R.I. Enterprises, New Berlin, WI.

Samples of StarLink and Pioneer 3751 (non-StarLink control) field grain were shipped to F.R.I. Enterprises where masa was prepared. The corn/lye mixture was heated to boiling and left simmering at about 91°C for one hour. The masa was allowed to stand and steep overnight and the grain was then rinsed with 24°C water. The grain was agitated and hand cleaned to remove any loosely attached but soft bran material. The hydrated grain (about 48% moisture) was disc milled into dough (masa) and used for preparing subsequent cooked products.

Tortillas were prepared by cold pressing and baking on a hot plate at approximately 196°C to mimic the use of a three-pass industrial oven. Taco shells were prepared by placing cold-pressed masa on a taco shell rack preheated to 218°C. The tortillas were baked in a 218°C oven for 24 minutes. Tortilla chips were prepared by cold pressing masa and cutting rectangular chips from the disc. The chips were heated for two minutes at 193°C, then fried in Canola oil at 188°C. Finish drying was done in a microwave oven.

Corn Puffs (Extruded Snacks), and Corn "Ringed" Cereal

Corn puffs and puffed cereal were prepared by Dr. Triveni Shukla, F.R.I. Enterprises, New Berlin, WI.

Samples of degermed corn meal obtained from StarLink and the control Pioneer grain were provided to F.R.I. Enterprises by the GLP Processing Program facility of Texas A&M University. An extrusion process that simulates a commercial process was used to prepare puffed corn snacks. No additives were used in the composition. Samples of extruded breakfast cereal were prepared from the corn meal using a similar process to that used to prepare corn puffs. No other ingredients were added to the corn meal and water.

Puffed Corn Snacks (Extruded Snacks)

Thomas Diehl, Diehl, Inc., Defiance, OH, also prepared puffed corn snacks. Samples of degermed corn meal obtained from StarLink and the control Pioneer grain were provided to Diehl, Inc. by the GLP Processing Program facility of Texas A&M University.

Puffed corn snacks (corn curls) were prepared by an extrusion process which simulates a commercial process using a pilot scale extruder located at Ohio State University. The extrusion samples were cut to commercial-type lengths and dried in a cabinet dryer for a short period of time to remove moisture down to a desired level of about 10%.

Corn Flakes

Corn flakes were prepared by Dr. Malcolm Gerngross, GLP Processing Program, Texas A&M University, College Station TX, using a small pilot scale process.

Degermed grit samples were prepared at Texas A&M University by dry grinding samples of StarLink and control Pioneer 3751 grain. The formulation consisted only of grits and water, with no other ingredients added. The grits were soaked in water and then cooked with steam in a pressure cooker at 15 – 18 psi for 16 minutes. Grits were dried at maximum temperatures of approximately 68°C and then sealed in a plastic bag at ambient temperature and allowed to equilibrate overnight. Prior to flaking, a sufficient amount of steam was applied to the grits for a period of less than one minute to make their surface area sticky. The grits were then fed slowly through flaking rolls, and placed in a preheated oven (between 274 and 302°C) and toasted for 2.5 minutes.

Polenta

Polenta was prepared by Debbie Lohmeyer of the National Food Laboratory, Dublin CA, using a small pilot scale process.

Samples of polenta were prepared from degermed corn meal samples supplied by the GLP Processing Program of Texas A&M University. The recipe for the polenta was adapted from James McNair's Favorites cookbook (McNair and Moore, 1999).

Chicken broth was added to a large metal stockpot. The pot was placed on a large burner, and the chicken broth was brought to a boil over high heat. The corn meal was whisked in slowly. The mixture was cooked for 36 minutes with moderate heat and constant stirring with a flat wooden spoon. The pot was removed from the heat, and parmesan cheese was stirred in. Stirring continued for 5 minutes until the cheese was melted. The mixture was poured into a pan, which had been lightly coated with cooking spray and allowed to cool.

Hush Puppies

Hush puppies were prepared by Debbie Lohmeyer of The National Food Laboratory, Dublin CA, using a small pilot scale process.

Samples of hush puppies were prepared from degermed corn meal samples supplied by the GLP Processing Program of Texas A&M University. The recipe for the hush puppies was adapted from Justin Wilson's Home Grown Louisiana Cooking cookbook (Wilson, 1990).

In a Hobart mixer with bowl and paddle, sifted all purpose wheat flour, salt, baking soda, baking powder, garlic powder and pepper were mixed for 30 seconds on low speed. Corn meal and green onions were added and mixed for 30 seconds. In a separate bowl, the eggs and milk were combined and poured into the corn meal mixture, with mixing for 1 minute. Hot vegetable

oil was added and mixed for 1 minute. Using an ice cream scoop #50, the batter was dropped into corn oil previously heated to 177°C and cooked until golden brown (2 minutes). The hush puppies were drained on paper towels.

Corn Muffins and Corn Bread

Corn muffins and corn bread were prepared by Debbie Lohmeyer of The National Food Laboratory, Dublin, CA, using a small pilot scale process.

Samples of corn bread and corn muffins were prepared from degermed corn meal samples supplied by the GLP Processing Program of Texas A&M University. The recipe for the corn muffins and corn bread was adapted from James McNair's Favorites cookbook (McNair and Moore, 1999).

Corn meal, all purpose wheat flour, sugar, baking powder and salt were combined and mixed on low speed for 2 minutes in a Hobart mixer with bowl and paddle attachment. The eggs, oil and milk were combined in a separate bowl and whisked by hand for 1 minute. The wet ingredients were poured into the Hobart bowl containing the dry ingredients, with the mixer running on low speed, and mixed for 1 minute. For corn bread, the mixture was poured into an 8" x 8" baking pan (sprayed lightly with cooking spray), and for muffins, the mixture was scooped with a large ice cream scoop into each muffin tin lined with a paper baking cup (with the cup being about three fourths full). Baking time was 20 minutes for the corn bread and 15 minutes for the muffins.

4. EXPERIMENTAL PROCEDURES

4.1 Protein Extraction for ELISA

Each sample was ground with dry ice for 1-3 minutes as required until well ground. The steepwater concentrate and hull samples were not ground. Two approximately 1g sub-samples of each sample were extracted for ELISA analysis and each extract was assayed twice to provide a total of four determinations.

If more than one laboratory prepared a specific finished food product (e.g., tortilla chips), then each was sampled and tested as indicated above. When one laboratory made two different batches of the same finished food product (e.g., corn bread), then each batch was sampled and tested as indicated above and the values were reported for each batch separately. In some cases, the ELISA analysis was repeated because either the values of two different batches of the same food showed large differences (e.g., corn muffins), or the values were greater than 2000 ppb.

4.2 Immunoassay

Two ELISA methods were used to determine the amount of Cry9C protein in the extracts. Both are Sandwich Enzyme Linked ImmunoSorbent Assays based on the specific interaction between antibody and antigen, each utilizing polyclonal antibodies.

Aventis (in-house) ELISA:

The in-house Aventis ELISA used in this study is based on a polyclonal capture antibody developed by Strategic Diagnostics Inc., and an in-house polyclonal detection antibody supplied by Aventis CropScience N.V. (Gent, Belgium). Both antibodies were raised against the Cry9C protein produced in bacteria, which has an amino acid sequence identical to that found in the transgenic StarLink plants. The method described is a modification of Aventis method BAM/005/00, in which the polyclonal goat anti-Cry9C detection antibody of method BAM/003A/99 is substituted for the monoclonal mouse anti-Cry9C antibody described in Biotechnology Support Method BAM/005/00 and supplied as part of the SDI Cry9C ELISA kit. Utilizing a polyclonal antibody improves the sensitivity for denatured proteins.

The reference substance was a purified sample of the same Cry9C protein as that used to produce the antibodies, and was also obtained from Aventis CropScience N.V. (Gent, Belgium). Both the capture antibody and the Cry9C antiserum used as a detection antibody in this ELISA detect both denatured and intact Cry9C protein. Experience with the use of these antibodies in ELISA assays suggests that the present assay is capable of detecting 0.47 ppb (4.7 ppb fresh weight of sample) Cry9C protein in an extract, and is probably quantitative above 1 ppb (10 ppb fresh weight of sample). Since the EnviroLogix ELISA assay (see next section) was deemed to be more sensitive, the validation of the Aventis ELISA was not completed at this time.

Cry9C ELISA plates were prepared using the SDI capture antibody (Rabbit anti-Cry9C polyclonal antibody; Strategic Diagnostics Inc., Part No.: 1020113) according to SOP BT-6005.01. Plates were stored in sealed bags containing desiccant at approximately 4°C and then warmed to room temperature before use. The plate system using the capture antibody is essentially identical to the plates offered commercially by Strategic Diagnostics, Inc., Newark, DE, USA, as ELISA kit part number 7110030.

Samples were extracted in standard extraction buffer (SEB). SEB buffer is 50 mM TrisHCl, 100 mM KCl, 5% v/v glycerol, 10 mM EDTA, 10 mM EGTA, 14 mM 2-Mercaptoethanol, 1 mM Benzamidine HCl, 5 mM s-amino n-caproic acid, 1 µg/mL Antipain, 1 µg/mL Leupeptin, and 1 mM PMSF in water, pH 7.0. The PMSF is added just before use.

Serially diluted sample extracts were applied to blocked ELISA plates at 100 µL/well followed by incubation on a shaker at 500 rpm for 60 ± 15 minutes at room temperature. Any Cry9C protein present in the samples was bound to the capture antibody. Unbound material was removed by rinsing the wells six times with deionized water.

The plate was subsequently incubated under the same conditions with the goat anti-Cry9C detection antibody, followed by five rinses with deionized water. The plate was then incubated in the same way with a third, anti-goat, antibody conjugated to horseradish peroxidase and then rinsed the same way.

A peroxidase substrate, Tetramethylbenzidine (TMB), was then added and converted by the peroxidase to a blue product in proportion to the amount of Cry9C protein present in the sample. The reaction was stopped with 0.5 M H_2SO_4, and the color changed to yellow. The resulting color development was measured in a microplate reader (Molecular Devices THERMOmax) at 450 nm.

The EnviroLogix ELISA:

The EnviroLogix ELISA kit (catalog No. AP 008, see Appendix 5) was also used to determine Cry9C protein concentrations. The kit uses polyclonal antibodies. The antibodies in this kit were raised against the Cry9C protein produced in bacteria, which has an amino acid sequence identical to that found in the transgenic StarLink plants. The High Sensitivity Protocol, as described in the manufacturer's package insert (Appendix 5), was used in this study.

The reference substance was a purified sample of the same Cry9C protein as that used to produce the antibodies and was obtained from Aventis CropScience N.V. (Gent, Belgium). The capture polyclonal antibody and the polyclonal detection antibody in this ELISA detect both denatured and intact Cry9C protein. The LOD of the kit according to the manufacturer is 0.07 ppb Cry9C protein in an extract of grain, flour meal and grits. As 5 mL of buffer is used to extract 1 g of sample, this corresponds to an LOD of 0.35 ppm Cry9C protein in the samples. The LOD for solvent extracted germ was 6.42 ppb in our laboratory. The limit of quantitation (LOQ) in these matrices is given in the kit instructions as 1.5 ppb. During validation of the method using the matrices in this report, the LOQ values ranged from 1-2.5 ppm in the Aventis laboratory for most matrices. The LOQ values are reported in Table 4. Taco shells (5ppb) and solvent extracted germ (30ppb) had higher LOQ's than other matrices.

Samples were extracted in EnviroLogix extraction buffer, pH 10.0 (the buffer components are a trade secret of EnviroLogix, Inc.). Serially diluted sample extracts were applied to blocked ELISA plates at 100 µL/well followed by incubation on a shaker at 500 rpm for thirty minutes at room temperature. Any Cry9C protein present in the samples was bound to the capture antibody. Unbound material was removed by rinsing the wells four times with washing buffer.

The plate was subsequently incubated under the same conditions with the conjugated second antibody, for two hours, followed by four rinses with wash buffer. The Substrate, which consists of Tetramethylbenzidine (TMB), was then added and converted by the peroxidase to a blue product in proportion to the amount of Cry9C protein present in the sample. The reaction was allowed to proceed for 30 minutes, and stopped with 0.5 M H_2SO_4, and the color changed to yellow. The resulting color development was measured in a microplate reader (Molecular Devices THERMOmax) at 450 nm.

4.3 Limit of Detection and Limit of Quantitation of EnviroLogix Immunoassay

Validation for each matrix type was carried out using the non-StarLink samples. Values below the LOD are reported as ND (Non-detectable) and values below the LOQ but above the LOD are reported as '<LOQ'.

4.3.1 LOD

The LOD is determined for each matrix type using the average standard curve and the concentration derived from the background optical density (OD) of the negative control samples. The LOD is the concentration corresponding to an OD value three standard deviations above the mean background OD, or the LOD specified by the manufacturer, whichever is the higher. For the EnviroLogix kit, the manufacturer defines the LOD as at least 0.07 ppb in the extract (0.35 ppb in the samples).

The limit of detection is expressed in the unit of concentration (ng/mL) and the unit of weight ratio (ng/g matrix, i.e. ppb) calculated based on the extraction of 1 g of matrix per 5 mL extraction buffer for the EnviroLogix kit (Table 1). An ELISA reading giving rise to a Cry9C concentration above this limit of detection level is assumed to be greater than the zero dose reading.

4.3.2 Validation

The Cry9C ELISA procedure was validated for each matrix type by using the non-StarLink samples. For the EnviroLogix ELISA, non-StarLink control samples of each matrix type were each fortified at 0.2, 0.5, 1 and 3 ng/mL in the extraction buffer prior to extraction, corresponding to 1, 2.5, 5 and 15 ng/g sample (Appendix 4)

The LOQ is given by the lowest concentration of the standard that meets the criteria for the LOQ. Validity criteria are (a) analyte recoveries from fortified matrix samples are ≥ 60 % and ≤ 130 % and (b) the coefficient of variance (relative standard deviation) is less than 25%. When the nature of a specific matrix or the effect of a process causes a lower recovery, the lowest concentration of the standard that gives a smaller coefficient of variance than 25% is used as the LOQ. The recovery was less than 60% in some matrices, at all concentrations tested, and thus the LOQ was determined in those cases as the concentration which gave a coefficient of variance smaller than 25%.

Table 4: Limit of Detection (LOD) and Limit of Quantitation (LOQ) in Cry9C EnviroLogix ELISA of Control Samples

Process	Commodity	EnviroLogix method			
		LOD (ng/mL)	LOD (ng/g, ppb)	LOQ (ng/mL)	LOQ (ng/g, ppb)
	Whole Corn	0.07	0.35	0.5	2.5
Dry Mill	Degermed Meal	0.07	0.35	0.5	2.5
	Degermed Flour	0.07	0.35	0.5	2.5
Wet Mill	Starch	0.07	0.35	0.2	1
	Gluten	0.07	0.35	0.5	2.5
	Solvent extracted Germ*	0.65	6.42	3.0	30
	Refined Oil	0.07	0.35	0.2	1
Processed Foods	Soft Tortillas	0.07	0.35	0.2	1
	Tortilla chips	0.07	0.35	0.2	1
	Baked Taco Shells	0.07	0.35	1	5
	Puffed Cereal	0.07	0.35	0.2	1
	Corn Puffs	0.07	0.35	0.5	2.5
	Corn Flakes	0.07	0.35	0.2	1
	Hush Puppies	0.07	0.35	0.5	2.5
	Corn Muffins	0.07	0.35	0.5	2.5
	Corn Bread	0.07	0.35	0.5	2.5
	Polenta	0.07	0.35	0.5	2.5
	Masa (dough)	0.07	0.35	0.2	1
	Commercial Tacos	0.07	0.35	0.5	2.5

(footnotes on next page)

Footnotes to table 4:
LOD = Limit of Detection
LOQ = Limit of Quantitation
* Solvent extracted germ was extracted in 10 mL of buffer per gram of sample, due to absorption of the buffer by the matrix, whereas all other matrices were extracted in 5 mL of buffer per gram as per the manufacturer's instructions.

The LOQ values shown are those that were attained using the EnviroLogix kit in the Aventis laboratory with the sample matrices tested. These values (1-2.5 ppm in the sample) are below those given by the manufacturer (7.5 ppm), even for the grain, meal and flour, which are the matrices specified by the manufacturer. An exception was the LOD and LOQ for solvent extracted germ. The non-StarLink sample gave a high background signal, leading to an LOD of 15.1 ppb. The LOQ was estimated to be in the range of 30ppb in the matrix.

4.4 Protein Determination

The Bradford assay method was used to determine the concentration of total extractable protein (TEP) in extracts used for ELISA assays. The assay relies on the binding of the dye, Coomassie blue G250, to protein. The TEP was determined for each sample extract (Appendix 3) in order to show that protein was being extracted from the samples during the extraction process.

5. CALCULATIONS

Cry9C content (Cry9C ELISA)

SoftMax Pro™ software (Molecular Devices, Version 1.2.0) was used to derive the concentration of immunoreactive Cry9C protein. The optical density (OD) values were adjusted for the buffer blank. The optical density was converted to the Cry9C protein concentration using the standard curve. The ELISA data given in Tables 2 and 3 are each the average of four determinations (duplicate assays on duplicate samples).

6. STATISTICAL ANALYSIS

Descriptive statistics (mean, standard deviation, and coefficient of variance) were calculated for each sample matrix and treatment.

7. RESULTS AND DISCUSSION

Wet milled and dry milled corn fractions and various processed food products were made from 100% StarLink grain and control non-StarLink grain. Each fraction was analyzed for Cry9C protein levels by two different quantitative ELISA methods. One was a commercial kit manufactured by EnviroLogix, Inc., and the other was an Aventis in-house ELISA. Both utilize two polyclonal anti-Cry9C antibodies.

Yellow dent grain is primarily used for animal feeds (60%) and exports (20%), and only about 20% is used for food or industrial purposes. Of the 20% that is used for food, most of the grain that is processed goes through wet milling (about 75%), which almost exclusively uses yellow dent grain

(EPA, 2001). Dry milled and masa-based products make up the remainder of the processed corn products, which uses both yellow and white corn types. StarLink, which contains the Cry9C protein, has only been incorporated into yellow corn varieties.

7.1 Assay comparison

The amounts of Cry9C protein detected in the various finished corn products were in general higher using the EnviroLogix method than the Aventis ELISA method, but overall the two methods produced very similar quantitative values for each matrix tested. It is not surprising to see differences from one ELISA method (EnviroLogix and Aventis) to another, especially when the total protein levels and the Cry9C levels are so very low (ppm to ppb). These assay differences reflect differences in the extraction buffers and methods used. In two samples ("ringed" cereal, starch), the Aventis method detected higher concentrations of Cry9C protein than did the EnviroLogix method. When the Cry9C protein was detected in one method, it was generally detected in the other method as well, ensuring that either method has sufficient sensitivity to detect the presence of the Cry9C protein. The only exception to this is found in just 2 samples where the Cry9C protein level was extremely low – one of the Fried Tortilla chip samples (made by Texas A&M) and the corn puffs (produced by Diehl). The EnviroLogix ELISA measured 20.3 ppb in the chips and 4.6 ppb in the puffs, while the levels measured in the Aventis ELISA were below the detection limit.

Both test methods appear to be sensitive and specific, and therefore not prone to false negative or false positive results. However, since the EnviroLogix method detected higher levels of the Cry9C protein in most matrices, Aventis would recommend it as the method of choice, especially since it is available commercially. Much of the discussion below will focus on the preferred EnviroLogix ELISA results. The results from both ELISA methods are presented in Tables 7, 8 and 9. The actual concentration of the Cry9C protein, expressed in ppb, are given in these tables along with a calculated percentage of the amount of the Cry9C protein remaining in the various fractions and food products as compared to the level of the Cry9C protein in grain. Thus the percentage of Cry9C protein in whole grain is defined as 100%. A graphical summary of the EnviroLogix ELISA data is also presented in Figures 1, 2, 3 and 4, displaying the average Cry9C protein levels along with the standard deviations. In cases where more than one sample of a particular processed food was prepared and tested, the highest level was included in these graphs. For example, two facilities made tortilla chips (Texas A&M and FRI) and FRI made two different batches, but only the highest result from one of the three samples, 20.3 ppb, is included in the graphics. The data used to create Figures 1 through 4 are the same, but products were grouped together according to the method of processing. In Table 4, only the finished food products were included to allow for a more precise presentation of the data and a comparison across all types of processing and cooking.

Small-scale processors make every effort to mimic commercial scale production, but generally the commercial processes would be harsher on proteins than the small-scale processing methods. For example, some Masa processes uses longer boiling or alkali steeping times than were used in this study. Thus, while the Cry9C protein levels are indeed low, the use of these small-scale processors likely to cause an overestimate of the amount that would be found if 100% StarLink grain were processed commercially.

7.2 ELISA analysis – Wet Milled corn products

Corn products produced from wet milling are used for either food products (i.e., starch and refined oil) or animal feed products (i.e., gluten, hulls, steepwater and solvent extracted germ). As a simple description, the wet milling process separates starch and oil from the protein components of the corn kernel. Wet milled starch is the starting material for the production of sweeteners, alcohol and industrial starch. The refined oil is used in food, feed and industrial products. The remaining wet milled fractions are generally high in protein content and utilized primarily for animal feeds (May, 1987).

The levels of the Cry9C protein in wet milled corn products are provided in Table 7. Both the actual concentration of Cry9C found and the percentage that it represents of the raw StarLink grain are shown for each sample. Although there was variation between the two ELISA methods, the overall trends were consistent.

The Cry9C protein content determined for the raw StarLink whole grain was typical and in the middle range for StarLink grain. The wet milled gluten, hulls, solvent extracted germ and steepwater concentrate samples retained relatively high amounts of the Cry9C protein (Table 7), which is expected since these fractions are high in total protein content. The Cry9C protein data were consistent with a previous processing experiment (Shillito, 1998).

The starch produced by wet milling had extremely low levels of the Cry9C protein, at about 13 ppb. The total crude protein (soluble and insoluble) level in this sample was 0.5% (Personal Communication, Malcolm Gerngross, Texas A&M), based on a total nitrogen determination. This is somewhat higher than the typical acceptable industry values of 0.30-0.35% for residual protein levels. Therefore this small-scale process probably overestimates the actual amount of Cry9C protein that would be found in commercially produced wet milled starch derived from 100% StarLink grain. No Cry9C protein was detected in the wet milled bleached/deodorized (refined) oil. The results are not surprising since refined oil contains the lowest amount of extractable protein (Appendix 3) of any corn fraction tested.

The Cry9C protein levels, along with the standard deviation values, are also graphically presented in Figure 1. The wet milled fractions utilized for animal feed and those for human food consumption are indicated. A comparison of the Cry9C protein levels across all finished food products for wet milling, dry milling and Masa processing is provided in Figure 4.

The results obtained for the food fractions, starch and oil, coincide with the calculations by the EPA in "White Paper on the Possible Presence of Cry9C Protein in Processed Human Foods made from Food Fractions Produced through the Wet Milling of Corn" (Table 5). The EPA calculated that corn starch made from 100% StarLink grain would contain about 1.61×10^{-2} µg/g (or 16.1 ppb) Cry9C protein and that corn starch would contain approximately 0.01% protein (0.1 mg protein/gram starch). Aventis data, using the EnviroLogix ELISA method, show that starch prepared from 100% StarLink grain contains approximately 13 ppb Cry9C protein and that the starch samples contain 0.10 mg/g and 0.16 mg/g extractable protein (StarLink and control grain respectively, Appendix 3). For refined oil, the extractable protein levels are also low, measuring 0.13 mg/g and 0.17 mg/g for StarLink and control grain, respectively, with no detectable amounts of the Cry9C protein.

Table 5: Comparison of calculated and actual Cry9C protein and total extractable protein levels

Wet milled fraction	EPA calculations[1]		Aventis Data	
	Cry9C protein	TEP	Cry9C Protein[2]	TEP[3]
NT[4] refined oil	NA[5]	0.10 mg/g oil	ND	0.17 mg/g oil
StarLink refined oil	ND[6]	0.10 mg/g oil	ND	0.13 mg/g oil
NT starch	NA	0.10 mg/g starch	ND	0.16 mg/g starch
StarLink starch	16.1 ppb	0.10 mg/g starch	13.2 ppb	0.10 mg/g starch

[1] EPA, 2001
[2] EnviroLogix ELISA method (see Table 7)
[3] TEP = total extractable protein; values are given in Appendix 3.
[4] NT = Non-StarLink
[5] NA = Not applicable
[6] ND = Not detected

7.3 ELISA analysis – Masa Processed fractions and food products

The Cry9C protein level is dramatically reduced in Masa (dough) produced by alkali treatment of corn kernels, to only 126.6 ppb (Table 8). This represents more than a 99.5% loss of the Cry9C protein from the starting 100% StarLink grain prior to using this material for preparing tortilla type finished food products.

Three different types of tortilla products were made from the Masa – soft tortillas, fried tortilla chips, and baked taco shells, and these products were made at two different processing facilities. Of the six different samples tested, all produced from 100% StarLink grain, the Cry9C protein was detected in only two of the samples, at 23.6 and 20.3 ppb. The Cry9C protein level was below the detection limit in the remaining samples.

The Cry9C protein levels, along with the standard deviation values, are also graphically presented in Figure 2. The products that are cooked are indicated. A comparison of the Cry9C protein levels across all finished food products for Masa processing, wet milling, and dry milling is provided in Figure 4.

Three factors appear to cause destruction of the Cry9C protein – heat, shear or pressure, and alkali treatment. The processing and cooking methods used for the preparation of tortilla type products are fairly harsh, using alkali treatment, high heating temperatures and pressure from grinding. Bt proteins, including the Cry9C protein, are known to be soluble at alkali pHs (MacIntosh et al., 1990) and a significant fraction of the Cry9C may be washed away during the production of the Masa and subsequent tortilla products. The temperatures for frying ranged from 188°C to 193°C and for baking ranged from 196°C to 320°C.

7.4 ELISA analysis – Tortilla shell products from grocery store shelves

In a previous study entitled, "Analysis of Taco shells for Cry9C protein" (Shillito, 2000), Kraft "Taco Bell Home Originals" taco shells obtained from 19 commercial sources were analyzed for presence of the Cry9C protein using an Aventis in-house ELISA based on antibodies developed by

Strategic Diagnostics, Inc. Seven of the batches have been previously tested by PCR and found to be positive for the presence of StarLink-derived DNA.

No detectable Cry9C protein was found in baked taco shells (Sample #449F) produced from 100% StarLink grain in this study. While the taco shells tested in the previous study were all below the detection limit of the Aventis ELISA using SDI antibodies, a subset of these samples were retested using the more sensitive Envirologix ELISA method (Table 6).

Table 6: Cry9C in Taco shells from commercial sources

Aventis Sample ID or BTID:	Cry9C Protein level (ppb ± SD)	PCR result[1]
AV00-010214	ND	Positive
AV00-010215	4.3 ± 0.12	Positive
AV00-010216	2.5 ± 0.09	Positive
AV00-010217	1.7 ± 0.06	Positive
AV00-010220	2.0 ± 0.22	Positive
AV00-010221	ND	Not tested
AV00-010225	ND	Positive
AV00-010229	<LOQ	Positive
491A	ND	Not tested

[1] from MRID 452402-02 (2000)
ND = Not detected.
<LOQ = less than the LOQ, the LOQ is 2.5ppb.

Samples AV00-010221 and 491A were commercial taco chips prepared from white corn, and these were used as negative control samples. Sample 491A was used in validation of the EnviroLogix assay for this matrix.

Of the seven PCR-positive tortilla samples, four tested above the level of quantitation with ranges from 1.7 to 4.3 ppb. Of three other samples that were positive by PCR, one showed trace levels of Cry9C (<LOQ), and the other two showed no detectable Cry9C. The disagreement between the PCR and protein tests could be due to the sensitivity of the PCR test, or may be due to sampling issues, as a different taco shell from the same packet was taken for the each protein test than for the PCR test.

The results from all of the tortilla samples, both those known to be made from 100% StarLink grain and those collected from grocery store shelves in the fall of 2000, demonstrates that the Cry9C protein level is either below the detection limit, or extremely low, at ppb levels. The amount of StarLink in the grain used for production of the grocery store taco products was not known, but because Cry9C protein was found, the amount of the StarLink grain used in the taco shell production must have been above 20 ppb. These Cry9C protein levels, above 20 ppb, would not be found in commercial tortilla products produced today, due to the grain testing program which has been put in place at the grain elevators and mills by the USDA Grain Inspection, Packers and Stockyard Administration (GIPSA-FGIS directive 9181.1, and bulletin #191).

7.5 ELISA analysis – Dry milled corn products

The dry milling process produced corn meal and corn flour for use in a variety of finished food products listed in Table 8. The Cry9C protein levels in grain, degermed corn meal and corn flour were the same, indicating that this particular method of processing has little effect on the Cry9C protein level.

Snacks (corn puffs) and cereal ("ringed" and corn flakes) made from corn meal contained either no detectable Cry9C protein or levels that were extremely low, approximately 4.5 ppb. This represents more than a 99.9% loss of the Cry9C protein from the starting StarLink grain.

Corn bread, corn muffins, polenta, and hush puppies all had considerably higher levels of Cry9C protein than found in all the other finished food products. The Cry9C protein levels ranged from 483 to 2636 ppb. When the Cry9C protein level is expressed as a percentage of the starting level in grain, these products ranged from 3.4% to 18.4%, with corn bread and hush puppies on the high end. Therefore, more than 93% of the Cry9C protein is lost during the preparation of polenta and corn muffins while approximately 82% is lost for corn bread and hush puppies.

The Cry9C protein levels, along with the standard deviation values, are also graphically presented in Figure 3. The products that are cooked are indicated. A comparison of the Cry9C protein levels across all finished food products for dry milling, wet milling and Masa processing is provided in Figure 4.

Data on corn bread, corn muffins, polenta, and hush puppies allow for some interesting comparisons of the contribution of the recipe dilution with that of the cooking methods to the overall loss of the Cry9C protein (see Figure 5). For instance, while the recipe ingredients are the same for corn bread and corn muffins, the baking times and surface to volume ratio during the baking is quite different. The corn muffins were baked for 15 minutes at 204°C in muffin tins and the corn bread was baked for 20 minutes in a 8" x 8" pan at the same temperature. (Note: Each batch of corn muffins and corn bread was identified with a separate sample number and a corn muffin sample was a homogenized whole corn muffin.).

During the initial testing of these two batches, the levels of the Cry9C protein for corn bread were consistent at 2254 and 2281 ppb, but the levels measured in corn muffin from the two batches batches were quite different, at 283 and 1350 ppb. During a second set of testing, initiated due to the relatively high levels found in the corn bread and the divergent values for the corn muffins, the corn bread samples again produced very similar Cry9C protein levels, at 2469 ppb and 2264 ppb, giving an average of 2361 ppb and 2273 ppb, respectively for 456M and 456N. However, the values for corn muffins were again quite divergent. The corn muffins contained 283 ppb and 463 ppb Cry9C. Thus muffins from batch 456I contained 283 ppb and 1065 ppb, giving an average of 674 ppb, while muffins from batch 456H contained 1350 ppb and 463 ppb, giving an average of 906 ppb. The variability of the Cry9C protein level between individual muffins may be related to the position of the muffin within the muffin pan. In any case, the Cry9C protein levels in corn muffins are less than half that measured in the corn bread, implying that there may be greater heat transfer causing a greater loss of the Cry9C protein in the relatively small volume of a corn muffin.

If the Cry9C protein values are averaged across corn bread and muffins, only about 15% of the Cry9C protein is destroyed during baking for corn bread, while more than 70% is destroyed during muffin baking.

Hush puppies contained the highest Cry9C protein level, probably due to the relatively high level of corn (48% wt/wt) used in this recipe. Hush puppies are fried for 2 minutes in hot oil at 177°C and the percentage of the Cry9C protein dropped by about 65% of the initial value. The amount of corn in polenta is much lower, at 11.6% and the polenta is cooked near boiling for about 40 minutes, leaving only 3.7% of the initial level of the Cry9C protein, or a reduction of about 70% of the Cry9C protein due to cooking.

It should be further noted that Cry9C protein levels in uncooked mixes for corn bread, corn muffins, hush puppies and polenta would certainly be higher than that found in the cooked products. To estimate the level of the Cry9C protein in uncooked mixes, the dark bars (Estimated % due to the amount of corn in the recipe, Figure 5) could be used.

8. CONCLUSION

This study was undertaken for two purposes. The first was to determine the amount of Cry9C protein that could be detected after processing in wet and dry milled fractions and masa processed fractions and other processed foods that were made from 100% StarLink corn. The second objective was to compare ELISA (Enzyme Linked ImmunoSorbent Assay) methods, and validate determine which ELISA method is the most sensitive for detecting the presence or absence of the Cry9C protein in food ingredients or finished products.

The grain used for this study consisted of grain harvested from a field planted to 100% StarLink™ hybrid corn. Food products that had a wide range of different processing and cooking conditions were prepared. Some foods were prepared at more than one facility and, in some cases, more than one batch of a food was prepared in order to ensure that representative samples from typical processing methods and recipes were prepared. In all cases, the processing of fractions and the food preparation were done to mimic real commercial practices as much as possible. Small-scale processors make every effort to mimic commercial scale production, but generally the commercial processes would be harsher on proteins than the small-scale processing methods.

Although ELISA methods are very sensitive they are also more expensive, take more time and are more difficult to perform than another immunologically based method, the lateral flow strip test (Strategic Diagnostics, Inc. and EnviroLogix, Inc.). An ELISA test is generally performed in a well equipped laboratory setting by trained laboratory personnel. Strip tests, on the other had, are quick and easy to perform and can detect one StarLink™ kernel in a sample of 800 kernels or about 20 ppb of Cry9C protein. Strip tests are simple to perform in most any environment, field, lab, processing facility and grain elevators, by personnel with minimal training.

The two ELISA methods produced very similar quantitative values for each matrix tested, however, the amounts of Cry9C protein detected in the various finished corn products were in general higher when using the Envirologix method.

This study does reveal the impact of processing on the fate of the Cry9C protein in finished foods. The results demonstrate that there is extensive reduction in the amount of detectable Cry9C protein during processing of 100% StarLink™ grain into a range of processed corn food products. All processing methods reduce the amount of Cry9C protein significantly. The degree of the reduction depends on the specific processing method used. three factors appear to cause destruction of the Cry9C protein. These are heat, shear or pressure, and alkali treatment. The loss of Cry9C protein is

due to a combination of recipe dilution, processing methods and cooking. The greater the dilution and the more harsh the processing/cooking (heat, shear or pressure and alkali treatment), the lower the level of the Cry9C protein in the finished food product.

The Cry9C protein levels detected in these finished foods represent a worst case scenario, for two reasons. First, the foods in question were made from 100% StarLink grain. Foods made from 100% StarLink™ grain are not available to consumers in the marketplace. Second, the foods tested were produced in small scale processing rather than by commercial processing and therefore are likely to overstate the amount of Cry9C that would remain after harsher commercial processing methods were employed.

The wet milled production of starch from 100% StarLink™ grain led to the loss of more than 99.9% of the Cry9C protein and no Cry9C was detected in the refined oil. A further reduction in the Cry9C protein level in high fructose corn syrups is likely because syrup products are processed from wet milled starch. These findings are consistent with the calculated values given by the EPA in their White Paper on Wet Milling (EPA, 2001).

Foods produced by the Masa process were also extremely low in Cry9C protein. Cry9C protein was detected, at 23.6 and 20.3 ppb, in only two of the six different samples tested. The amount of Cry9C protein was below the detection limit in the remaining samples. Seven samples which had been collected from grocery store shelves in September 2000 during a recall of taco shells, and that had tested positive for *cry9C* DNA were also assayed. Very low levels of the Cry9C protein (1 to 4 ppb) were detected in four of the six PCR-positive taco shell products, and one sample contained a trace amount (<LOQ) of Cry9C. The other two samples were below the detection limit of the ELISA assay. These Cry9C protein levels would not be found in commercial tortilla products produced today, due to the grain testing program which has been put in place at the grain elevators and mills by the USDA Grain Inspection, Packers and Stockyard Administration (GIPSA-FGIS directive 9181.1, and bulletin #191). This program utilizes lateral flow strips, as described above, with a sensitivity level of about 20 ppb.

Less than 5 ppb of the Cry9C protein was found in corn snacks and cereal corn products produced using the degermed corn meal fraction of 100% StarLink™ grain. The highest Cry9C protein levels, ranging from approximately 450 to 2700 ppb, were detected in polenta, corn bread, corn muffins and hush puppies. While these levels are based on cooked products, the Cry9C protein levels in uncooked mixes for corn bread, corn muffins, hush puppies and polenta would certainly be higher, and perhaps as much as three times the values determined from the cooked products.

9. REFERENCES

No.	Doc. No.	Report No.	Author(s). Year. Title. Source. Edition. Pages.
1.		CM00Q009 supplemental	**DeLisle, A. (2000)** Further studies on detection of CBH351 DNA in taco shells: (Investigations carried out between the afternoon of September 28 and the morning of September 29, 2000.) MRID 452402-02
2.			**Environmental Protection Agency (March 7, 2001)** EPA Analysis of the Impact of Wet Milling on the Cry9c Protein Content in Food. Federal Register Vol 66. #64, pp 17706-17708.
3.			**MacIntosh, S., Stone, T., Sims, S., Hunst, P., Greenplate, J., Marrone, P., Perlak, F., Fischhoff, D., and Fuchs, R. (1990)** Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. *J. Invert. Path.* 56:258-266.
4.			**May, J.B. (1987)** Wet Milling: Process and Products, in Corn: Chemistry and Technology, Eds. S.A. Watson & P.E. Ramstad, Amer. Ass. Cereal Chem., pgs. 377-397.
5.			**McNair, J.K., and Moore, A. (1999)** James McNair's Favorites, ISBN: 0811801152, Chronicle Books, September, 1999.
6.			**Orr, D., (2001)** GIPSA-FGIS directive 9181.1 January 26, 2001, Testing for StarLink corn - lateral flow test strip method; and bulletin #191, Revision to the Starlink testing and certification procedures, February 2, 2001.
7.	B002580	CM98B003	**Shillito, R.D. (1998)** Phosphinothricin acetyltransferase and Cry9C protein content in processed fractions of transgenic field corn event CBH351, USA, MRID 450257-01
8.	B003088	CM00B011	**Shillito, R.D. (2000)** ELISA analysis of Cry9C Protein in CBH351 StarLink™ Corn subject to Pilot Scale Alkaline Processing. MRID 452753-01

9.	B003065	CM00Q009	**Shillito, R.D., Artis, T. (2000)** Analysis of Taco shells for Cry9C protein, MRID 452464-01
10.			**Wilson, J. (1990)** Justin Wilson's Homegrown Louisiana Cooking, ISBN: 0026301253, MacMillan Publishing Company NY, June, 1990.

Table 7: Cry9C in wet milled corn products derived from 100% StarLink and Control Grain

BTID[1]	Sample Description	EnviroLogix ELISA ppb Cry9C[2]	Percent of Cry9C as compared to that in Whole Grain	Aventis ELISA ppb Cry9C	Percent of Cry9C as compared to that in Whole Grain
454A	NT[3] Whole Grain	ND[4]		ND	
455A	StarLink Whole Grain	14,275 ± 640	100	9184 ± 954	100
454B	NT Wet Milled Starch	ND	-	ND	-
454C	NT Wet Milled Gluten	ND	-	ND	-
454D	NT Wet Milled Hull	ND	-	ND	-
454E	NT Steepwater Concentrate	ND	-	ND	-
457A	NT Solvent Extracted germ	ND	-		
457B	NT Refined Oil	ND	-	ND	-
455B	StarLink Wet Milled Starch	13.2 ± 0.65	0.09	24.4 ± 1.81	0.26
455C	StarLink Wet Milled Gluten	4,063 ± 124	28.4	1,354 ± 190	14.7
455D	StarLink Wet Milled Hull	12,950 ± 324	90.7	9,738 ± 559	106
457C	StarLink Solvent Extracted Germ	25,650 ± 723.4	180	11,496 ± 1556	125
455E	StarLink Steepwater Concentrate	1,588 ± 149	11.1	1,204 ± 160	13.1
457D	Wet Milled Bleached/Deodorized StarLink Oil	ND	-	ND	-

1. BTID: Sample identification number
2. ppb: Parts per billion
3. NT: Non-StarLink control sample
4. ND: Not detected

Table 8: Cry9C in Masa-based food products derived from 100% StarLink and Control Grain

BTID[1]	Sample Description	EnviroLogix ELISA	Percent of Cry9C in	Aventis ELISA	Percent of Cry9C in
		ppb Cry9C[2]	Whole Grain	ppb Cry9C	Whole Grain
454A	NT Whole Grain[3]	ND[4]		ND	
455A	StarLink Whole Grain	14,275 ± 640	100	9,184 ± 954	100
418D	NT Masa (dough) A&M	ND		ND	
418N	NT Soft Tortillas, A&M[5]	ND		ND	
451A	NT Soft Tortillas, FRI[6]	ND		ND	
449C	NT Baked Taco Shells, FRI	ND		ND	
418M	NT Fried Tortilla chips A&M	ND		ND	
449A	NT Fried Tortilla Chips, FRI	ND		ND	
449B	NT Fried Tortilla Chips, FRI	ND		ND	
414C	StarLink Masa (dough) A&M	127 ± 2.2	0.89	51.8 ± 7.1	0.56
414A	StarLink Soft Tortillas, A&M	23.6 ± 1.4	0.17	6.5 ± 1.2	0.07
451B	StarLink Soft Tortillas, FRI	ND	-	ND	-
449F	StarLink Baked Taco Shells, FRI	ND	-	ND	-
414B	StarLink Fried Tortilla Chips, A&M	20.3 ± 1.7	0.14	ND	-
449D	StarLink Fried Tortilla Chips, FRI	ND	-	ND	-
449E	StarLink Fried Tortilla Chips, FRI	ND	-	ND	-

[1] BTID: Sample identification number
[2] ppb: Parts per billion
[3] NT: Non-StarLink control sample
[4] ND: Not detected
[5] A&M: samples produced at Texas A&M University
[6] FRI: samples produced at FRI.

Table 9: Cry9C in dry milled processed food products derived from 100% StarLink and Control Grain.

BTID[1]	Sample Description	EnviroLogix ELISA	Percent of Cry9C in	Aventis ELISA	Percent of Cry9C in
		ppb Cry9C[2]	Whole Grain	ppb Cry9C	Whole Grain
454A	NT Whole Grain[3]	ND[4]		ND	
459A	NT dry milled meal	ND		ND	
461A	NT dry milled flour	ND		ND	
455A	StarLink Whole Grain	14,275 ± 640	100	9,184 ± 954	100
459B	StarLink dry milled corn meal	15,075 ± 417	106	6,348 ± 748	69
461B	StarLink dry milled flour	15,363 ± 747	108	7,621 ± 1062	83
450B	NT Corn Puffs, FRI	ND	–	ND	–
452A	NT Corn puffs, Diehl	ND	–	ND	–
450A	NT Puffed Cereal, FRI	ND	–	ND	–
453A	NT Corn Flakes, A&M	ND	–	ND	–
456A	NT Polenta	ND	–	ND	–
456B	NT Polenta	ND	–	ND	–
456F	NT Corn Muffins	ND	–	ND	–
456G	NT Corn Muffins	ND	–	ND	–
456K	NT Corn Bread	ND	–	ND	–
456L	NT Corn Bread	ND	–	ND	–
456E	NT Hush Puppies	ND	–	ND	–
450D	StarLink Corn Puffs, FRI	ND	-	ND	-
452B	StarLink Corn Puffs, Diehl	4.6 ± 0.1	0.03	ND	-
450C	StarLink "Ringed" Cereal, FRI[6]	4.5 ± 0.4	0.03	13.9 ± 2.2	0.15
453B	StarLink Corn Flakes, A&M[5]	ND	-	ND	-
456C	StarLink Polenta	483 ± 32.5	3.4	219 ± 31.4	2.4
456D	StarLink Polenta	645 ± 93.7	4.5	302 ± 41.2	3.3
456I	StarLink Corn Muffins	674 ± 422	4.7	76 ± 10.5	0.83
456J	StarLink Corn Muffins	906 ± 475	6.4	275 ± 15.4	3.0
456M	StarLink Corn Bread	2,361 ± 206[7]	16.5	322 ± 56.1	3.5
456N	StarLink Corn Bread	2,273 ± 194[7]	15.9	1,257 ± 162	13.7
456H	StarLink Hush Puppies	2,636 ± 158[7]	18.4	1,163 ± 55.7	12.7

[1] BTID: Sample identification number
[2] ppb: Parts per billion
[3] NT: Non-StarLink control sample
[4] ND: Not detected
[5] A&M: samples produced at Texas A&M University
[6] FRI: samples produced at FRI.
[7] Because of the relatively high values observed in the corn muffins, corn bread and hush puppies, each was sampled and assayed on two different dates. Values represent an average of the two assay dates. The high SD's reflect the large difference between samples. SD's within each sample were between 2.2 and 9.7% of the sample means.

Figure 1: Cry9C protein levels in wet milled food fractions and products made from 100% StarLink Grain

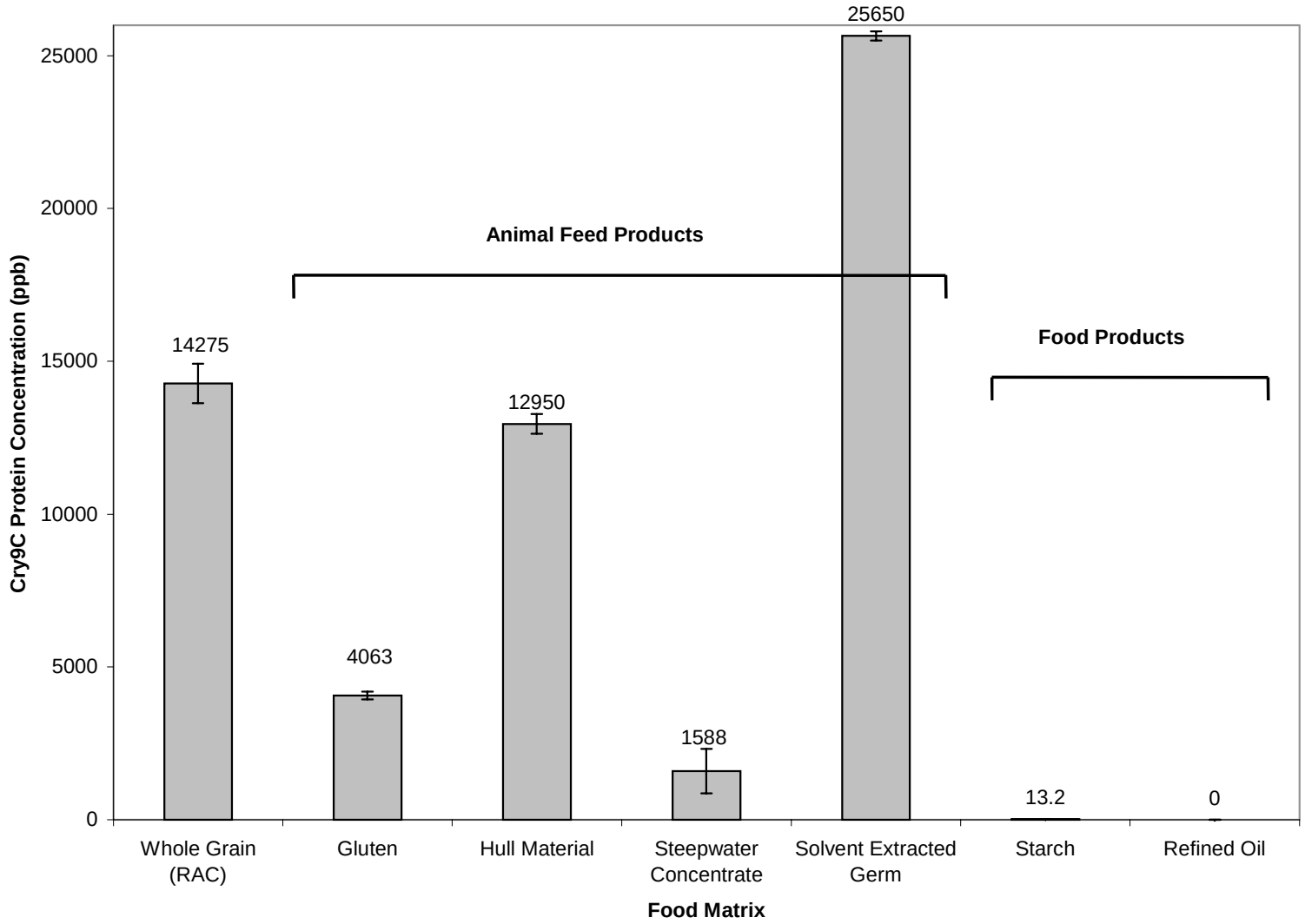


Figure 2: Cry9C protein levels in Masa processed fractions and products made from 100% StarLink Grain

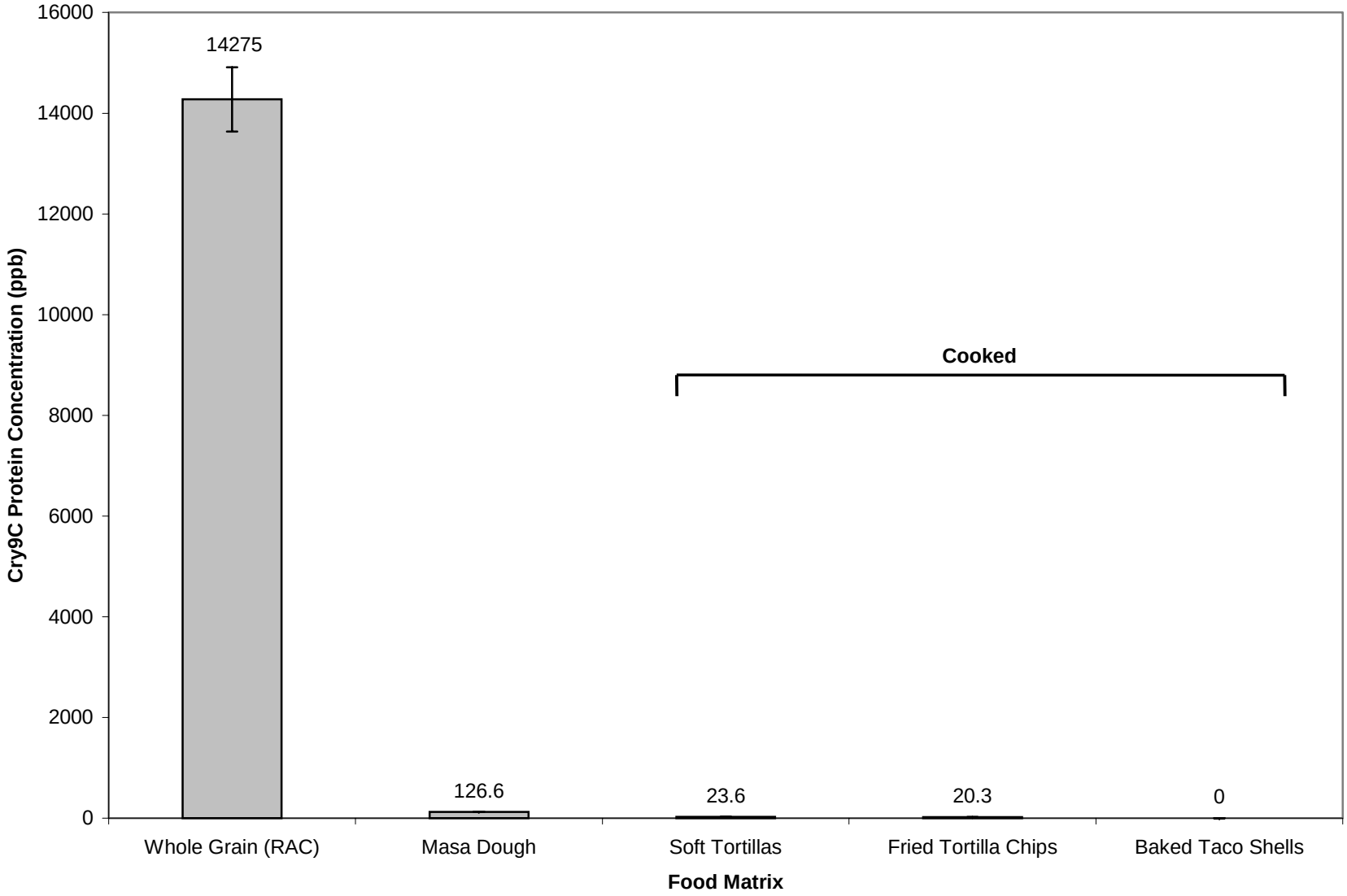


Figure 3: Cry9C protein levels in Dry Milled fractions and products made from 100% StarLink Grain

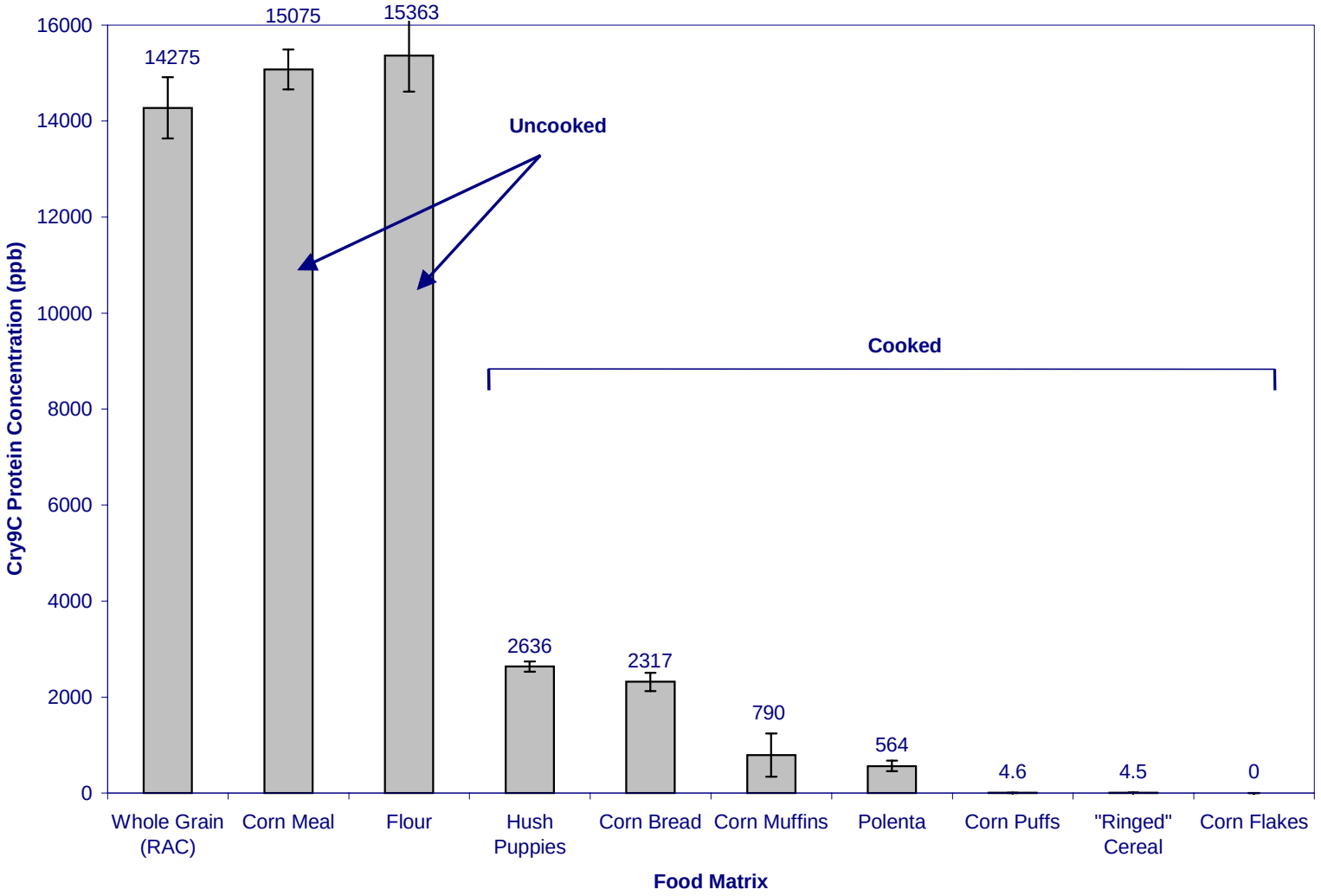


Figure 4: Cry9C protein levels in all finished food products made from 100% StarLink Grain

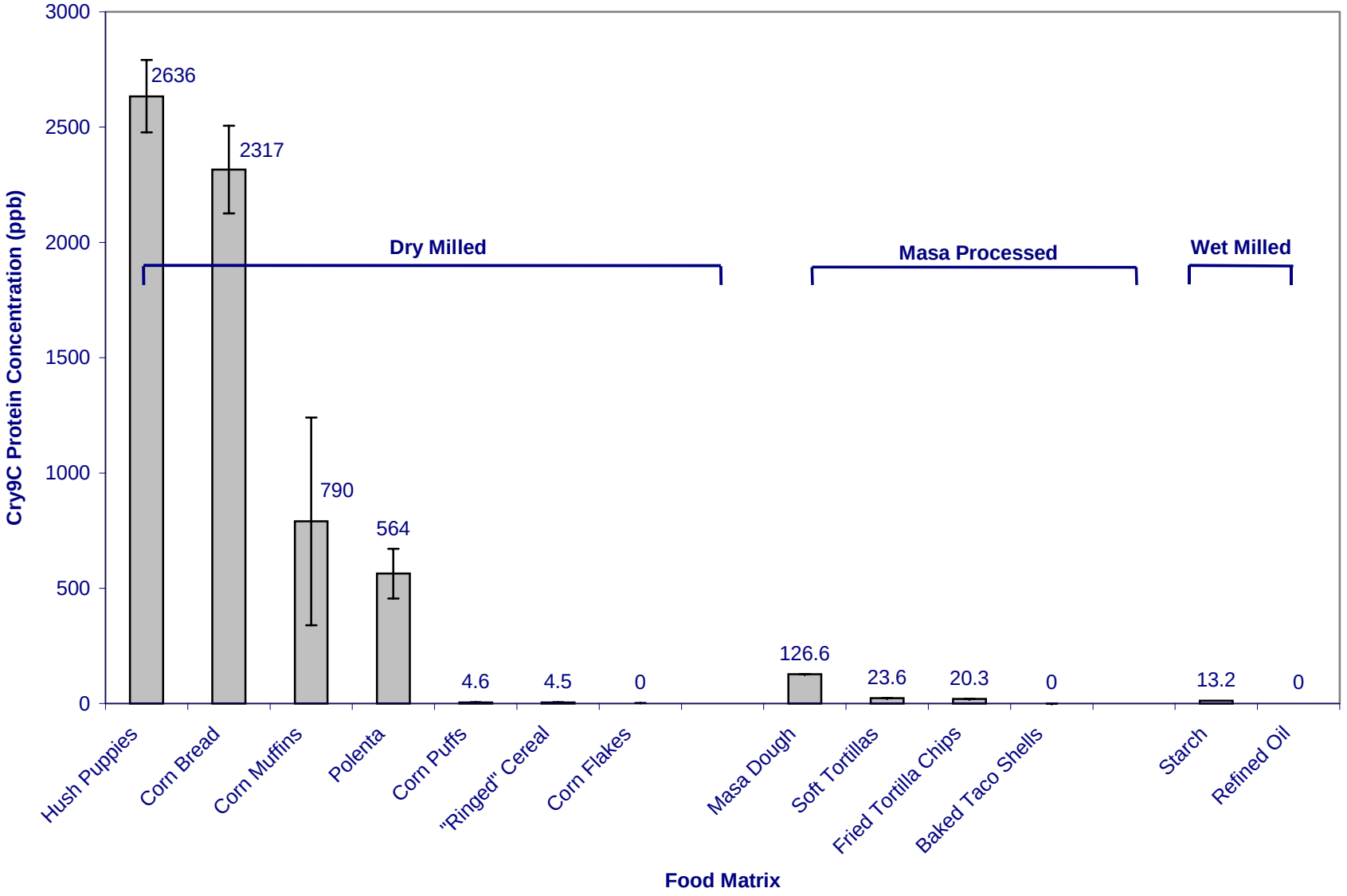
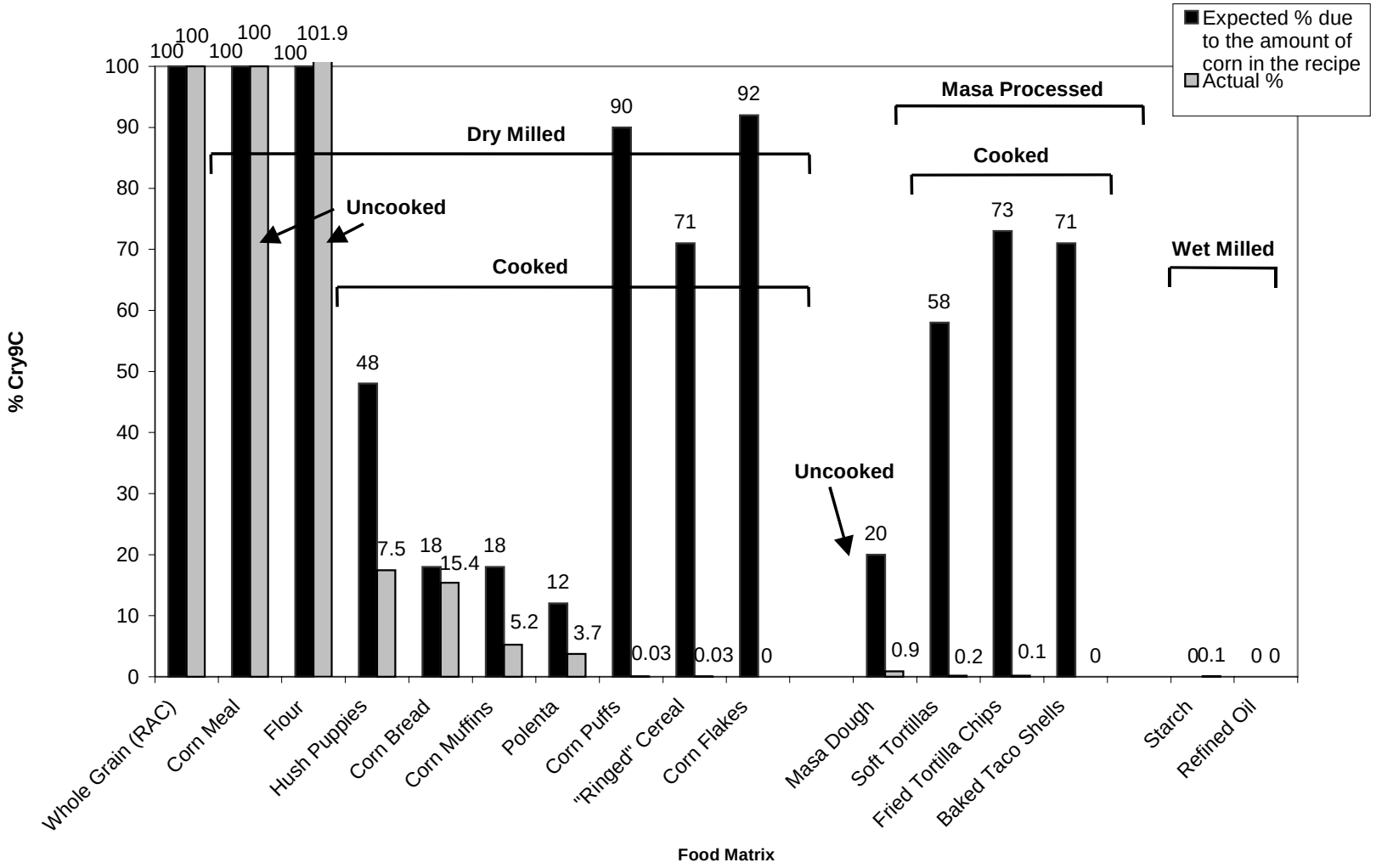


Figure 5: Effects of recipe dilution and processing of Cry9C protein remaining in foods made from 100% StarLink Grain



Appendix 1: Preparation of Processed Food Items and wet milled fractions.

The following processed food products were prepared directly from 100% StarLink grain and Pioneer 3751 (non-StarLink control) grain, or from fine corn meal produced during dry milling, as appropriate; Masa (dough), tortillas, tortilla chips by Texas A&M University; corn puffs, "ringed" cereal, soft tortillas by F.R.I. Enterprises; corn puffs by Diehl, Inc.; corn flakes by Texas A&M; tortilla chips and taco shells by F.R.I. Enterprises; and polenta, hush puppies, corn muffins and corn bread by The National Food Laboratory (Table A3-1).

Table A3-1: Sample List

Product:	**Prepared from:**	**Prepared by:**	**Assigned BTID:**	
			Prepared from Control	**Prepared from StarLink**
Whole Grain			454A	455A
Corn Meal	Whole Grain (dry milled)	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	459A	459B
Corn Flour	Whole Grain (dry milled)	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	461A	461B
Wet Milled Starch	Whole Grain (wet milled)	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	454B	455B
Wet Milled Gluten	Whole Grain (wet milled)	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	454C	455C
Wet Milled Hull	Whole Grain (wet milled)	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	454D	455D
Steepwater Concentrate	Whole Grain (wet milled)	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	454E	455E
Solvent extracted germ	Whole Grain (wet milled)	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	457A	457C
Refined Oil	Whole Grain (wet milled)	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	457B	457D
Masa (dough)	Whole Grain	Texas A&M University College Station, TX (Dr. Lloyd Rooney)	418D	414C
Tortillas (soft)	Whole Grain	Texas A&M University College Station, TX (Dr. Lloyd Rooney)	418N	414A

Product:	**Prepared from:**	**Prepared by:**	**Assigned BTID:**	
			Prepared from Control	**Prepared from StarLink**
Tortilla chips (fried)	Whole Grain	Texas A&M University College Station, TX (Dr. Lloyd Rooney)	418M	414B
"Corn puffs"	Corn Meal	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	450B	450D
"Ringed" cereal ("Cheerios-like")	Corn Meal	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	450A	450C
Tortillas (soft)	Whole Grain	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	451A	451B
"Corn puffs"	Corn Meal	Diehl, Inc. Defiance, OH (Tom Diehl)	452A	452B
Corn Flakes	Whole Grain (grits)	Texas A&M University College Station, TX (Dr. Malcolm Gerngross)	453A	453B
Tortilla chips (fried)	Whole Grain	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	449A, B	449D, E
Taco shells (baked)	Whole Grain	F.R.I. Enterprises New Berlin, WI (Dr. Triveni Shukla)	449C	449F
Polenta	Corn Meal	The National Food Laboratory Dublin, CA (Debbie Lohmeyer)	456A, B	456C, D
Hush puppies	Corn Meal	The National Food Laboratory Dublin, CA (Debbie Lohmeyer)	456E	456H
Corn muffins	Corn Meal	The National Food Laboratory Dublin, CA (Debbie Lohmeyer)	456F, G	456I, J
Corn bread	Corn Meal	The National Food Laboratory Dublin, CA (Debbie Lohmeyer)	456K, L	456M, N

1. Wet Milled Fractions

The Food Protein Research laboratory, College Station, TX Station under the supervision of Malcolm Gerngross produced wet milled fractions. The wet milled commodities produced for analysis were hulls, steepwater concentrate, gluten, solvent extracted germ, starch, and bleached, deodorized (refined) oil.

Samples were handled in a manner that minimizes the possibility of contamination. It is the policy of Texas A&M Food Protein Research laboratory to use only containers and utensils washed with detergent and rinsed with water.

The wet milling process used by the Food Protein Research laboratory is as follows:

The whole grain samples are dried in a Proctor Schwartz oven between 54-71°C. The final moisture content after drying is between 10-15%. The light impurities are separated using a Kice aspirator. After aspiration, the sample is screened in a Vac-Away two screen cleaner. Large and small foreign particles (screenings) are separated from the grain.

The cleaned grain is steeped in 49-54°C water containing 0.1-0.2% sulfur dioxide (sulfurous acid) for 22-48 hours. At the end of the steeping period, the whole grain is passed through a Bauer mill with devil toothed plates and a majority of the germ and hull are removed using a hydroclone. Germ and hull are dried at 74-91°C to obtain final moisture between 5-10%. After drying, the germ and hull are separated using aspiration.

The cornstock (without germ and hull) is ground in a Rietz mill with a 0.023" screen. The material going through the 0.023" screen is passed through a Dynascreen equipped with a 43-micron screen. Material on top of the screen is a product of batch processing and is discarded. In commercial industry, only bran (hull material) remains on top of the screen. The process water (with starch and gluten) passing through the 43-micron screen is separated into component parts using batch centrifugation.

The germ is moisture conditioned to 12%, heated to 88-104^{0}C, flaked in a Ferrell-Ross flaking roll with a gap setting of 0.008 to 0.012", and pressed in a Rosedown expeller to liberate part of the crude oil. Resulting fractions are expelled crude oil and press cake with residual crude oil.

The press cake is placed in stainless steel batch extractors and submerged in 49-60°C solvent (hexane). After 30 minutes, the hexane is drained and fresh hexane added to repeat the cycle two more times. The final two washings are for 15-30 minutes each. After the final draining, warm air is forced through the extracted press cake to remove residual hexane.

The miscella (crude oil and hexane) is passed through a Precision Scientific Recovery unit to separate the crude oil and hexane. Crude oil is heated to 73-90°C for hexane removal.

The crude oil recovered from expelling and solvent extraction is combined, samples, and refined according to AOCS method Ca9a52. After refining, the refined oil and soap stock is separated.

Comparison to Industrial Practice:

The grain was wet milled in a way that simulates industrial practice as closely as possible. Because of compliance monitoring requirements and sample size, the samples were processed by batch rather than continuous, as in commercial operation.

2. Masa (Dough)
BTID No. assigned at Aventis CropScience: 418D (control), 414C (StarLink)

Prepared by: Dr. Lloyd Rooney Texas A&M University, College Station, TX

Production of Masa (dough), soft tortillas and tortilla chips via alkaline processing by Dr. Lloyd Rooney was reported previously in Aventis report CM00B011 (Aventis Document B003088, MRID 452753-01).

Alkaline Processing:
The grain was processed into finished foods at the Cereal Quality Lab, Texas A&M University, College Station, TX 77843-2474. Processing was performed under the supervision of Lloyd W. Rooney, Ph.D. The StarLink grain was processed on November 9-10, 2000, and the control grain was processed between November 13-15, 2000. The processing equipment was thoroughly cleaned between batches, and an additional sample of control grain was processed and discarded prior to preparation of the "official" control batch to further reduce the risk of contamination. The quantitative aspects (weights, times, temperatures, etc.) of the following processing steps pertain to processing of the StarLink grain batch. Processing parameters for the control grain batch were similar.

Prior to cooking, the grain was cleaned by aspiration and screening to remove impurities such as dust, chaff and weed seeds. The cooking/steeping process was conducted in a steam-jacketed stainless steel kettle. Twelve kg of the cleaned grain was divided among four nylon mesh bags (3 kg per bag). The bags were suspended into 50 liters of near-boiling water containing 120 g of pickling lime (essentially calcium hydroxide, or hydrated lime; commercial brand name "Mrs. Wages"). The quantity of lime used was approximately 1% of the grain weight in the batch. The addition of the grain to the cooking kettle was defined as "time zero" for the process. The lime-water mixture was stirred occasionally with a wooden stick to keep the lime in contact with the grain. Continued steam heating brought the temperature back to a simmering boil (about 97°C). The grain was then cooked for seven minutes at a low boil, with stirring about once every minute. After the seven-minute cooking time, the steam was turned off, and a lid was placed on the kettle to begin the steeping process. The grain was steeped (soaked) in the alkaline liquor overnight (about 15 hours). The temperature was not monitored for the process, but temperature profile data for this size batch are available from the Cereal Quality Lab.

The next morning, the alkali-cooked grain, or Nixtamal, was removed from the cooking kettle and washed with tap water in a bucket. The cooked kernels were hand-rubbed to remove most of the pericarp (hull material). The washing step removed the alkali and soluble material extracted from the kernels and much of the pericarp from the Nixtamal.

Production of Fresh Masa (Dough):
The washed Nixtamal was ground using a system of two matched carved stones, with one stone being stationary and the other mechanically rotated. The Nixtamal, still containing the germ, was conveyed through a center opening and into a gap between the stones. The Nixtamal was forced outward through grooves in the stones while being sheared and mashed to produce fresh masa. From the stone grinder, the fresh masa was run through steel rollers and formed into sheets. The sheet of masa was cut into disks during this process, with each masa disk weighing about 30 grams. Samples of the masa disks were frozen and shipped frozen to Aventis CropScience for analysis.

3. Soft Tortillas
BTID No. assigned at Aventis CropScience: 418N (control), 414A (StarLink)

Prepared by: Dr. Lloyd Rooney Texas A&M University, College Station, TX

The masa disks were transported from the sheeter on a conveyer belt to a triple pass gas-fired oven, where they were baked into tortillas. The baking time in the oven was about one minute. The baking temperatures within the three-tiered oven were about 320° C (top), 280° C (middle) and 240° C (bottom).

StarLink samples were shipped at ambient temperature, and the control samples were shipped frozen to Aventis CropScience for analysis. The samples were frozen upon arrival at Aventis.

4. Tortilla Chips
BTID No. assigned at Aventis CropScience: 418M (control), 414B (StarLink)

Prepared by: Dr. Lloyd Rooney Texas A&M University, College Station, TX

The soft tortillas were cooled for about 10 minutes and cut into sections ("triangles") prior to frying. The "triangle" sections were cooked in commercial corn oil in a commercial-type deep fat fryer. The temperature of the cooking oil was about 190°C and the cooking time was about 40 seconds. Six chips were prepared at a time in the cooker. The chips were shipped at ambient temperature to Aventis CropScience for analysis.

5. Soft Tortillas
BTID No. assigned at Aventis CropScience: 451A (control), 451B (StarLink)

Prepared by: Dr. Triveni Shukla, F.R.I. Enterprises, New Berlin, WI

Processing details:
Samples of StarLink and Pioneer 3751 (non-StarLink control) grain were shipped to F.R.I. Enterprises at the request of Aventis CropScience from Land O'Lakes Research Farm and Qualls Agricultural Laboratory, respectively.

Masa was prepared by mixing 1 kg of grain, 3 kg of water and 10 grams of calcium hydroxide (lime). After about 10 g of extraneous material was skimmed off, the mixture was heated to boiling and left simmering at about 91°C for one hour. The mass was allowed to stand and steep overnight. The steep was discarded, and the grain was rinsed with 1 kg of 24°C water in two portions. The grain was agitated and hand cleaned to remove any loosely attached but soft bran material. The hydrated grain (about 48% moisture) was disc milled into dough to be used for preparing subsequent cooked products.

Tortillas were prepared by cold pressing 55 grams of masa and baking on a hot plate at approximately 196°C to mimic the use of a three-pass industrial oven. The finished tortillas were stored under refrigerated conditions prior to shipping. The samples were shipped ambient by US Postal Express Mail and were received at Aventis CropScience on the following day.

6. Taco Shells
BTID No. assigned at Aventis CropScience: 449C (control), 449F (StarLink)

Prepared by: Dr. Triveni Shukla, F.R.I. Enterprises, New Berlin, WI

Processing details:
Masa (55 grams) prepared as described above was cold pressed and placed on a taco shell rack. Four flat discs were prepared at a time, and the rack was preheated to 218°C before receiving the cold tortilla discs. The tortillas on the rack were placed in a 218°C oven and baked for 24 minutes. The resulting taco shells were cooled and packaged for shipment to Aventis CropScience at ambient temperature.

7. Tortilla Chips
BTID No. assigned at Aventis CropScience: 449A,B (control), 449D,E (StarLink)

Prepared by: Dr. Triveni Shukla, F.R.I. Enterprises, New Berlin, WI

Processing details:
Masa (55 grams) prepared as described above was cold pressed into disc form. Rectangular chips were cut out from the disc. The chips were heated for two minutes at 193°C, then fried in canola oil at 188°C. Finish drying was done in a microwave oven to mimic many industrial operations.

The resulting chips were cooled and packaged for shipment to Aventis CropScience at ambient temperature.

8. Dry Milling to prepare corn meal

The 100% StarLink grain and the control Pioneer grain were degermed and processed into fine corn meal (approximately –30/+60 U.S. Standard Sieve size) and flour by the GLP Processing Program at the Food Protein Research laboratory, College Station, TX. This processing was performed under the supervision of Malcolm Gerngross.

Samples were handled in a manner that minimizes the possibility of contamination. It is the policy of Texas A&M Food Protein Research laboratory to use only containers and utensils washed with detergent and rinsed with water.

The dry milling process used by the Food Protein Research laboratory is as follows:

Processing Methods:

Whole grain is dried in a Proctor Schwartz oven at 54-71°C to moisture content of 10-15%. The light impurities are separated using a Kice aspirator. After aspiration, the sample is screened in a Vac-Away two screen cleaner to separate large and small foreign particles (screenings) from the grain.

The whole grain is moisture conditioned to 20-22% and allowed to “temper” for 2-2.5 hours. After tempering, the grain is impact milled in a Ripple mill. After milling, the cornstock is dried at 54-71°C for 30 minutes. Cornstock is allowed to cool to approximately 32°C after removal from the oven. The cornstock is passed over a 1/8” shaker screen. Material above the screen is further processed into large

grits, germ, and hull (bran). Material throughout the screen is separated into medium and small grits, coarse meal, meal, and flour.

The material above the 1/8" screen is passed through a Kice aspirator to separate the hull material and hull material with attached germ from the large grits and germ. The hull material and hull material with attached germ is aspirated at a lower setting to separate the hull material from the hull material with attached germ. Hull material with attached germ is passed through the Ripple mill and aspirated to separate the hull from the germ. The hull material is combined. Large grits and germ from the first aspiration are separated on an Oliver gravity separator. The germs are combined and dried at 54-71°C to about 7-10% moisture.

The material passing through the 1/8" shaker screen is separated using a Great Western sample sifter. The sifter is fitted with appropriate screen sizes to separate grits, meal and flour.

Grits and coarse meal were reground to increase the yield of flour and fine meal. Samples were shipped ambient to Aventis CropScience, and to food processors for processing into finished food products.

Comparison to Industrial Practice:

Dry milling by the GLP Program very closely simulates commercial dry milling practices. Slight variations in industrial milling practices are designed to suit the buyer's needs.

The majority of commercial plants will remove the oil from the germ by expelling (hard pressing). A small percentage will utilize direct solvent extraction to remove the crude oil. Due to equipment available to the GLP Program, hard pressing is not possible.

In comparison, the program's goal is to produce the same component parts for each sample within a study to be used in residue determination. Because of compliance monitoring requirements and sample size, the samples were processed by batch rather than continuous, as in commercial operation.

9. Corn Puffs (Extruded Snacks)
BTID No. assigned at Aventis CropScience: 450B (control), 450D (StarLink)

Prepared by: Dr. Triveni Shukla, F.R.I. Enterprises, New Berlin, WI

Processing details:
Samples of fine degermed corn meal (approximately –30/+60 U.S. Standard Sieve size) obtained from StarLink and the control Pioneer grain were provided to F.R.I. Enterprises by the GLP Processing Program facility of Texas A&M University.

Puffed corn snacks were prepared by an extrusion process, which simulates a commercial process. Adjusted moisture of the puffed snacks was about 14.5%, and no other additives were used in the composition. Process conditions were: Feed rate/hour = 300 lb.; Screw rpm = 300 – 310; Temperature = 188°C. Most of the water content flashed off during processing. Final moisture level of the puffed snacks was not determined.

The samples were shipped ambient to Aventis CropScience for analysis.

10. Corn "Puffed Cereal"
BTID No. assigned at Aventis CropScience: 450A (control), 450C (StarLink)

Prepared by: Dr. Triveni Shukla, F.R.I. Enterprises, New Berlin, WI

Processing details:
Samples of extruded breakfast cereal were prepared from the above degermed corn meal samples using a similar process. No other ingredients were added to the corn meal and water. Process conditions were: Feed rate/hour = 295 lb.; Screw rpm = 280; Temperature = 295 to 149°C.

The samples were shipped ambient to Aventis CropScience for analysis.

11. Corn Puffs (Extruded Snacks)
BTID No. assigned at Aventis CropScience: 452A (control), 452B (StarLink)

Prepared by: Thomas Diehl, Diehl, Inc., Defiance, OH

Processing details:
Samples of fine degermed corn meal (approximately –30/+60 U.S. Standard Sieve size) obtained from StarLink and the control Pioneer grain were provided to Diehl, Inc. by the GLP Processing Program facility of Texas A&M University.

Puffed corn snacks (corn curls) were prepared by an extrusion process which simulates a commercial process. The pilot scale extruder, located at Ohio State University, was preconditioned with the basic parameters of water injections set at about 20%, and temperature of ambient, 93°C, 124°C and 142°C F in the progressive barrel stages. A generic blend of corn meal was fed into the extruder, and the extrusion system was brought up to normal operating conditions. After acceptable extruded product was formed, the test corn meal samples were fed into the unit consecutively, with appropriate overlap times allowed before collecting the extruded samples. The extrusion samples were cut to commercial-type lengths and dried in a cabinet dryer for a short period of time to remove moisture down to a desired level of about 10%. After the test samples were extruded, the stock corn meal was again fed into the machine, temperatures in the barrel shut off, and water injection raised to the maximum level. This procedure cleaned out the barrels and screws of the extruder.

The finished samples and unused corn meal were shipped at ambient temperature to Aventis CropScience.

12. Corn Flakes
BTID No. assigned at Aventis CropScience: 453A (control), 453B (StarLink)

Prepared by: Dr. Malcolm Gerngross, GLP Processing Program, Texas A&M University, College Station, TX using a small pilot scale process.

Processing details:
Degermed grit samples were prepared at Texas A&M University by dry grinding samples of StarLink and control Pioneer 3751 grain. A grit size of approximately #4 (greater than 11/64 in) was used for the processing, and starting grit moisture was measured to be 14.7% for the control and 15.3% for StarLink grits. The formulation consisted only of grits and water, with no other ingredients added.

A weighed amount of grits from each sample was soaked in reverse osmosis water for 18 minutes to result in a moisture level of 22.4% for both samples. The product was cooked with steam in a pressure cooker at 15 – 18 psi for 16 minutes, with a maximum temperature of 125°C (control) and 124°C (StarLink). After the pressure cooking, any clumps of grits were broken up, and the moisture content of the grits was checked. The final moisture of the cooked grits was determined to be 31.4% (control) and 31.9% (StarLink), which was within the target moisture range of 28 to 32%. Cooking changed the appearance of the grits from hard, chalky white to a soft, translucent and light golden brown.

Grits were dried at maximum temperatures of 68°C (control) and 69°C (StarLink) until the moisture content was 22.5% (control) and 24.2% (StarLink). After the grits were removed from the dryer, they were promptly cooled for 5 minutes at a temperature of 4°C (control) and 4.5°C (StarLink). The grits were seated in a plastic bag at ambient temperature and allowed to equilibrate for approximately 19 hours (control) and 21.5 hours (StarLink).

Prior to flaking, a sufficient amount of steam (less than 5 psi) was applied to the grits for a period of less than one minute to make their surface area sticky. The grits were then fed slowly through flaking rolls, with a preset roll gap set at 0.004 of an inch. The rolls were set at 400 rpm, with no differential. A system to scrape the flakes off the rolls was in place.

The flakes (single layered on a stainless steel screen) were placed in a preheated oven and toasted for 2.5 minutes at a temperature between 274 and 302°C. The final moisture content of the toasted flakes was 5.7% (control) and 7.7% (StarLink). The finished flakes were packaged in wide mouth plastic containers and shipped to Aventis CropScience at ambient temperature by overnight express.

13. Polenta

BTID No. assigned at Aventis CropScience: 456A, 456B (control); 456C, 456D (StarLink)

Prepared by: Debbie Lohmeyer, The National Food Laboratory, Dublin, CA

Processing details:
Samples of polenta were prepared from degermed corn meal samples derived at the GLP Processing Program of Texas A&M University by dry grinding grain supplied by Aventis CropScience. The corn meal sample numbers were CM00B010-04-DM (control) and CM00B010-03-DM (StarLink). A standard recipe was selected, and its measurements were converted to a weight basis. Preparation of samples using the control or StarLink corn meal occurred on January 8, 2001.

Except for the corn meal, ingredients were purchased from a retail grocery store. Ingredient expiration dates/lot numbers were the same for the control and StarLink batches. Equipment used were the same for all batches. Exact mixing and cooking times were recorded for the first batch and used for subsequent batches to ensure uniformity of processing.

The recipe for the polenta was adapted from James McNair's Favorites cookbook. The recipe was converted to a weight basis using known conversion factors or by taking the weight average of 3 to 5 measurements on specific ingredients. Corn meal represents 11.6% of the ingredients by weight prior to cooking. The recipe used for the polenta follows.

Ingredients:
1893 grams chicken broth
258 grams corn meal
62 grams grated parmesan cheese

Preparation:
Chicken broth was added to a large metal stockpot. The pot was placed on a large burner, and the chicken broth was brought to a boil over high heat. The corn meal was whisked in slowly. The mixture was cooked for 36 minutes with moderate heat and constant stirring with a flat wooden spoon. The pot was removed from the heat, and parmesan cheese was stirred in. Stirring continued for 5 minutes until the cheese was melted. The mixture poured into a pan, which had been lightly coated with cooking spray, and allowed to cool. The recipe made one pan of polenta, with approximate dimensions of 12" x 9" x 2".

The samples were frozen and shipped frozen to Aventis CropScience for analysis.

14. Hush puppies

BTID No. assigned at Aventis CropScience: 456E (control), 456H (StarLink)

Prepared by: Debbie Lohmeyer, The National Food Laboratory, Dublin, CA

Processing details:
Samples of hush puppies were prepared from degermed corn meal samples derived at the GLP Processing Program of Texas A&M University by dry grinding grain supplied by Aventis CropScience. The corn meal sample numbers were CM00B010-04-DM (control) and CM00B010-03-DM (StarLink). A standard recipe was selected, and its measurements were converted to a weight basis. Corn meal represents 39% of the ingredients by weight prior to cooking. Preparation of samples using the control or StarLink corn meal occurred on January 8, 2001.

Except for the corn meal, ingredients were purchased from a retail grocery store. Ingredient expiration dates/lot numbers were the same for the control and 100% StarLink batches. Equipment used, including the oven, were the same for all batches. Exact mixing and cooking times were recorded for the first batch and used for subsequent batches to ensure uniformity of processing.

The recipe for the hush puppies was adapted from Justin Wilson's Home Grown Louisiana Cooking cookbook. The recipe was converted to a weight basis using known conversion factors or by taking the weight average of 3 to 5 measurements on specific ingredients. The recipe used for hush puppies follows.

Ingredients:

840 grams corn oil	0.7 grams ground cayenne pepper
115 grams all purpose flour	258 grams corn meal
6 grams salt	2 large eggs (123 grams total)
4 grams baking soda	121 grams milk
5.8 grams baking powder	27.2 grams hot vegetable oil
1.22 grams garlic powder	2 grams chopped green onions

Preparation:
A 3-quart saucepan was filled with corn oil and heated to 177°C. In a Hobart mixer with bowl and paddle, sifted flour, salt, baking soda, baking powder, garlic powder and pepper were mixed for 30 seconds on low speed. Corn meal and green onions were added and mixed for 30 seconds. In a separate bowl, the eggs and milk were combined and poured into the corn meal mixture, with mixing for 1 minute. The hot vegetable oil was added and mixed for 1 minute. Using an ice cream scoop, the batter was dropped into the hot corn oil and cooked until golden brown (2 minutes). The hush puppies were drained on paper towels. The recipe made 2 dozen hush puppies.

The samples were frozen and shipped frozen to Aventis CropScience for analysis.

15 Corn Muffins and Corn Bread

BTID No. assigned at Aventis CropScience: 456F, 456G (Muffins, control); 456I, 456J (Muffins, StarLink); 456K, 456L (Bread, control); 456M, 456N (Bread, StarLink)

Prepared by: Debbie Lohmeyer, The National Food Laboratory, Dublin, CA

Processing details:
Samples of corn bread and corn muffins were prepared from degermed corn meal samples derived at the GLP Processing Program of Texas A&M University by dry grinding grain supplied by Aventis CropScience. The corn meal sample numbers were CM00B010-04-DM (control) and CM00B010-03-DM (StarLink). A standard recipe was selected, and its measurements were converted to a weight basis. Preparation of samples using the control or StarLink corn meal occurred on January 8 and 9, 2001.

Except for the corn meal, ingredients were purchased from a retail grocery store. Ingredient expiration dates/lot numbers were the same for the control and 100% StarLink batches. Equipment used, including the oven, were the same for all batches. Exact mixing and cooking times were recorded for the first batch and used for subsequent batches to ensure uniformity of processing.

The recipe for the corn muffins and corn bread was adapted from James McNair's Favorites cookbook. The recipe was converted to a weight basis using known conversion factors or by taking the weight average of 3 to 5 measurements on specific ingredients. Corn meal represents 17.7% of the ingredients by weight prior to cooking. The recipe used for the corn bread and muffins follows.

Ingredients:
129 grams corn meal
115 grams all purpose flour
50 grams sugar
5.8 grams baking powder
6 grams salt
2 large eggs (123 grams total)
56 grams vegetable oil
242 grams milk

Preparation:
The oven was preheated to 204°C, and the racks were positioned so that the muffins or corn bread would bake in the middle of the oven. The corn meal, flour, sugar, baking powder and salt were combined and mixed on low speed for 2 minutes in a Hobart mixer with bowl and paddle attachment. The eggs, oil and

milk were combined in a separate bowl and whisked by hand for 1 minute. The wet ingredients were poured in to Hobart bowl containing the dry ingredients, with the mixer running on low speed, and mixed for 1 minute. For corn bread, the mixture was poured into an 8" x 8" baking pan (sprayed lightly with cooking spray), and for muffins, the mixture was scooped with a large ice cream scoop into each muffin tin lined with a paper baking cup (with the cup being about three fourths full). Baking time was 20 minutes for the corn bread and 15 minutes for the muffins. The recipe made one 8" x 8" corn bread or 1 dozen muffins.

The samples were frozen and shipped frozen to Aventis CropScience for analysis.

References:

1. Wilson, J. (1990) Justin Wilson's Homegrown Louisiana Cooking, ISBN: 0026301253, MacMillan Publishing Company NY, June, 1990.
2. McNair, J.K., and Moore, A. (1999) James McNair's Favorites, ISBN: 0811801152, Chronicle Books, September, 1999.

Appendix 2: Critical dates (NA = Sample did not require grinding)

Table A2-1: Critical dates for samples prepared from Non-StarLink grain (EnviroLogix ELISA method

Biotech Sample ID:	Matrix	Sample received	Sample ground	Sample extracted	Envirologix ELISA assayed	TEP assayed
454A	Whole Grain (RAC)	1/3/01	1/4/01	1/4/01	1/5/01	1/5/01
459A	Dry Milled Corn Meal	1/19/01	NA	2/19/01	2/20/2001; 2/21/2001	2/20/01
461A	Dry Milled Flour	1/26/01	NA	2/19/01	2/20/2001; 2/21/2001	2/20/01
454B	Wet Milled Starch	1/3/01	1/4/01	1/3/2001;1/22/2001	1/4/2001;1/23/2001	1/5/2001;1/23/2001
454C	Wet Milled Gluten	1/3/01	1/4/01	1/4/2001;1/22/2001	1/5/2001;1/23/2001	1/5/2001;1/23/2001
454D	Wet Milled Hull Material	1/3/01	1/4/01	1/4/01	1/5/01	1/5/01
454E	Steepwater Concentrate	1/3/01	1/4/01	1/4/01	1/5/01	1/5/01
457A	Wet Milled Solvent Extracted Germ	1/10/01	NA	1/10/2001;1/22/2001; 1/23/2001; 2/26/2001 3/22/2001	1/23/2001; 1/24/2001; 2/27/2001; 3/23/2001	1/23/2001; 1/24/2001; 2/27/2001; 3/23/2001
457B	Wet Milled Bleached Deodorized Oil	1/10/01	NA	1/10/01	1/11/01	1/11/01
418D	Masa (dough)	11/16/00	NA	2/21/01	2/22/01	2/22/01
451A	Soft Tortillas	12/12/00	1/2/01	1/2/01	1/3/01	1/3/01
418N	Soft Tortillas	11/17/00	1/2/01	1/2/01	1/3/01	1/3/01
418M	Fried Tortilla Chips	11/17/00	1/2/01	1/2/2001;1/22/01	1/3/2001;1/23/01	1/3/2001;1/23/01
449A	Fried Tortilla Chips	12/6/00	1/2/01	1/2/2001;1/22/01	1/3/2001;1/23/01	1/3/2001;1/23/01
449B	Fried Tortilla Chips	12/6/00	1/2/01	1/2/2001;1/22/01	1/3/2001;1/23/01	1/3/2001;1/23/01
449C	Baked Taco Shells	12/6/00	1/2/01	1/2/01	1/3/01	1/3/01
450B	Corn Puffs	12/7/00	1/2/01	1/2/01	1/3/01	1/3/01
452A	Corn Puffs	12/12/00	1/2/01	1/2/2001;1/22/2001	1/3/2001;1/23/2001	1/3/2001;1/23/2001
450A	Puffed cereal (FRI)	12/7/00	1/2/01	1/2/2001; 1/3/2001	1/3/2001; 1/4/2001	1/3/2001; 1/5/2001
453A	Corn Flakes	12/20/00	1/2/01	1/2/2001; 1/3/2001	1/3/2001; 1/4/2001	1/3/2001; 1/5/2001
456A	Polenta	1/9/01	NA	1/10/01	1/11/01	1/11/01
456B	Polenta	1/9/01	NA	1/10/01	1/11/01	1/11/01
456E	Hush Puppies	1/9/01	1/10/01	1/10/01	1/11/01	1/11/01
456F	Corn Muffins	1/9/01	1/10/01	1/10/01	1/11/01	1/11/01
456G	Corn Muffins	1/9/01	1/10/01	1/10/01	1/11/01	1/11/01
456K	Corn Bread	1/10/01	NA	1/10/01	1/11/01	1/11/01
456L	Corn Bread	1/10/01	NA	1/10/01	1/11/01	1/11/01

Table A2-2: Critical dates for samples prepared from 100% StarLink grain (EnviroLogix ELISA method)

Biotech Sample ID:	Matrix	Sample received	Sample ground	Sample extracted	Envirologix ELISA assayed	TEP assayed
455A	Whole Grain (RAC)	1/3/01	1/4/2001; 2/26/2001	1/4/2001; 2/26/2001	1/5/2001; 2/27/2001	1/5/2001; 2/27/2001
459B	Dry Milled Corn Meal	1/19/01	NA	2/19/2001; 2/26/2001	2/20/01; 2/21/01; 2/27/01	2/20/01; 2/27/2001
461B	Dry Milled Flour	1/26/01	NA	2/19/2001; 2/26/2001	2/20/01; 2/21/01; 2/27/01	2/20/2001; 2/27/2001
455B	Wet Milled Starch	1/3/01	1/4/01	1/3/2001;1/22/2001	1/4/2001;1/23/2001	1/5/2001;1/23/2001
455C	Wet Milled Gluten	1/3/01	1/4/01	1/4/01	1/5/01	1/5/01
455D	Wet Milled Hull Material	1/3/01	1/4/01	1/4/2001;1/22/2001	1/5/2001;1/23/2001	1/5/2001;1/23/2001
455E	Steepwater Concentrate	1/3/01	1/4/01	1/4/01	1/5/01	1/5/01
457C	Wet Milled Solvent Extracted Germ	1/10/01	NA	1/10/2001;1/22/2001	1/23/01	1/23/01
457D	Wet Milled Bleached Deodorized Oil	1/10/01	NA	1/10/01	1/11/01	1/11/01
414C	Masa (dough)	11/11/00	NA	2/21/01	2/22/2001; 2/23/2001	2/22/01
414A	Soft Tortillas	11/11/00	1/2/01	1/2/01	1/3/2001; 1/4/2001	1/3/01
451B	Soft Tortillas	12/12/00	1/2/01	1/2/01	1/3/01	1/3/01
414B	Fried Tortilla Chips	11/11/00	1/2/01	1/2/01	1/3/2001; 1/4/2001	1/3/01
449D	Fried Tortilla Chips	12/6/00	1/2/01	1/2/01	1/3/01	1/3/01
449E	Fried Tortilla Chips	12/6/00	1/2/01	1/2/01	1/3/01	1/3/01
449F	Baked Taco Shells	12/6/00	1/2/01	1/2/2001;1/22/2001	1/3/2001;1/23/2001	1/3/2001;1/23/2001
450D	Corn Puffs	12/7/00	1/2/01	1/2/2001;1/22/2001; 1/23/2001	1/3/2001;1/23/2001; 1/24/2001	1/3/2001;1/23/2001; 1/24/2001
452B	Corn Puffs	12/12/00	1/2/01	1/2/2001;1/22/2001; 1/23/2001	1/3/2001;1/23/2001; 1/24/2001	1/3/2001;1/23/2001; 1/24/2001
450C	Puffed cereal (FRI)	12/7/00	1/2/01	1/2/01	1/3/01	1/3/01
453B	Corn Flakes	12/20/00	1/2/01	1/2/2001;1/22/2001; 1/23/2001	1/3/2001; 1/24/2001	1/3/2001; 1/24/2001

Table A2-2: Critical dates for samples prepared from 100% StarLink grain (EnviroLogix ELISA method) continued

Biotech Sample ID:	Matrix	Sample received	Sample ground	Sample extracted	Envirologix ELISA assayed	TEP assayed
456C	Polenta	1/9/01	NA	1/10/01	1/11/2001; 1/12/2001	1/11/01
456D	Polenta	1/9/01	NA	1/10/2001;1/22/2001	1/11/2001; 1/12/2001;1/23/2001	1/11/2001;1/23/2001
456H	Hush Puppies	1/9/01	1/10/2001; 2/26/2001	1/10/2001; 2/26/2001	1/11/2001; 1/12/2001; 2/27/2001	1/11/2001; 2/27/2001
456I	Corn Muffins	1/9/01	1/10/2001; 2/21/2001	1/10/2001; 2/21/2001	1/11/2001; 1/12/2001; 2/22/2001; 2/23/2001	1/11/2001; 2/22/2001;
456J	Corn Muffins	1/9/01	1/10/2001; 2/21/2001	1/10/2001; 2/21/2001	1/11/2001; 1/12/2001; 2/22/2001; 2/23/2001	1/11/2001; 2/22/2001;
456M	Corn Bread	1/10/01	NA	1/10/2001; 2/21/2001	1/11/2001; 1/12/2001; 2/22/2001; 2/23/2001	1/11/2001; 2/22/2001;
456N	Corn Bread	1/10/01	NA	1/10/2001; 2/21/2001	1/11/2001; 1/12/2001; 2/22/2001; 2/23/2001	1/11/2001; 2/22/2001;

Table A2-3: Critical dates for commercial Taco samples (EnviroLogix ELISA method)

Aventis or Biotech Sample ID:	Matrix	Sample received	Sample ground	Sample extracted	Envirologix ELISA assayed	TEP assayed
AV00-010214	Taco Shells	9/26/00	3/19/01	3/19/01	3/20/01	3/21/01
AV00-010215	Taco Shells	9/26/00	3/19/01	3/19/01	3/20/01	3/21/01
AV00-010216	Taco Shells	9/26/00	3/19/01	3/19/01	3/20/01	3/21/01
AV00-010217	Taco Shells	9/26/00	3/19/01	3/19/01	3/20/01	3/21/01
AV00-010220	Taco Shells	9/26/00	3/19/01	3/19/01	3/20/01	3/21/01
AV00-010221	Taco Shells	9/26/00	3/19/01	3/19/01	3/20/01	3/21/01
AV00-010225	Taco Shells	9/26/00	3/19/01	3/19/01	3/20/01	3/21/01
AV00-010229	Taco Shells	9/26/00	3/19/01	3/19/01	3/20/01	3/21/01
491A	Taco Shells	3/19/01	3/19/01	3/19/01	3/20/01	3/21/01

Table A2-4: Critical dates for samples prepared from Non-StarLink grain (Aventis ELISA method)

Biotech Sample ID:	Matrix	Sample received	Sample ground	Sample extracted	Aventis ELISA assayed	TEP assayed
454A	Whole Grain (RAC)	1/03/01	1/04/01	1/05/01	1/05/01	1/05/01
459A	Dry Milled Corn Meal	1/19/01	NA	2/08/01	2/08/01	2/08/01
461A	Dry Milled Flour	1/26/01	NA	2/08/01	2/08/01	2/08/01
454B	Wet Milled Starch	1/03/01	1/04/01	1/4/2001; 1/25/2001	1/5/2001; 1/25/2001	1/5/2001; 1/25/2001
454C	Wet Milled Gluten	1/03/01	1/04/01	1/05/01	1/05/01	1/05/01
454D	Wet Milled Hull Material	1/03/01	1/04/01	1/05/01	1/05/01	1/05/01
454E	Steepwater Concentrate	1/03/01	1/04/01	1/05/01	1/05/01	1/05/01
457A	Wet Milled Solvent Extracted Germ	1/10/01	NA	1/11/01	1/12/01	1/12/01
457B	Wet Milled Bleached Deodorized Oil	1/10/01	NA	1/11/01	1/12/01	1/12/01
418M	Fried Tortilla Chips	11/17/00	1/02/01	1/04/01	1/05/01	1/05/01
451A	Soft Tortillas	12/12/00	1/02/01	1/04/01	1/05/01	1/05/01
418N	Soft Tortillas	11/17/00	1/02/01	1/04/01	1/05/01	1/05/01
449A	Fried Tortilla Chips	12/06/00	1/02/01	1/04/01	1/05/01	1/05/01
449B	Fried Tortilla Chips	12/06/00	1/02/01	1/04/01	1/05/01	1/05/01
449C	Baked Taco Shells	12/06/00	1/02/01	1/04/01	1/05/01	1/05/01
450B	Corn Puffs	12/07/00	1/02/01	1/04/01	1/05/01	1/05/01
452A	Corn Puffs	12/12/00	1/02/01	1/04/01	1/05/01	1/05/01
450A	Puffed cereal (FRI)	12/07/00	1/02/01	1/04/01	1/05/01	1/05/01
453A	Corn Flakes	12/20/00	1/02/01	1/04/01	1/05/01	1/05/01
456A	Polenta	1/09/01	NA	1/11/01	1/12/01	1/12/01
456B	Polenta	1/09/01	NA	1/11/01	1/12/01	1/12/01
456E	Hush Puppies	1/09/01	1/10/01	1/11/01	1/12/01	1/12/01
456F	Corn Muffins	1/09/01	1/10/01	1/11/01	1/12/01	1/12/01
456G	Corn Muffins	1/09/01	1/10/01	1/11/01	1/12/01	1/12/01
456K	Corn Bread	1/10/01	NA	1/11/01	1/12/01	1/12/01
456L	Corn Bread	1/10/01	NA	1/11/01	1/12/01	1/12/01

Table A2-5: Critical dates for samples prepared from 100% StarLink grain (Aventis ELISA method)

Biotech Sample ID:	Matrix	Sample received	Sample ground	Sample extracted	Aventis ELISA assayed	TEP assayed
455A	Whole Grain (RAC)	1/03/01	1/04/01	1/05/01	1/05/01	1/05/01
459B	Dry Milled Corn Meal	1/19/01	NA	2/08/01	2/08/01	2/08/01
461B	Dry Milled Flour	1/26/01	NA	2/08/01	2/08/01	2/08/01
455B	Wet Milled Starch	1/03/01	1/04/01	1/04/01	1/05/01	1/05/01
455C	Wet Milled Gluten	1/03/01	1/04/01	1/05/01	1/05/01	1/05/01
455D	Wet Milled Hull Material	1/03/01	1/04/01	1/05/01	1/05/01	1/05/01
455E	Steepwater Concentrate	1/03/01	1/04/01	1/5/2001; 1/25/2001	1/5/2001; 1/25/2001	1/5/2001; 1/25/2001
457C	Wet Milled Solvent Extracted Germ	1/10/01	NA	1/11/01	1/12/01	1/12/01
457D	Wet Milled Bleached Deodorized Oil	1/10/01	NA	1/11/2001; 1/25/2001	1/12/2001; 1/25/2001	1/12/2001; 1/25/2001
414A	Soft Tortillas	11/11/00	1/02/01	1/04/01	1/05/01	1/05/01
451B	Soft Tortillas	12/12/00	1/02/01	1/04/01	1/05/01	1/05/01
414B	Fried Tortilla Chips	11/11/00	1/02/01	1/4/2001; 1/25/2001	1/5/2001; 1/25/2001	1/5/2001; 1/25/2001
449D	Fried Tortilla Chips	12/06/00	1/02/01	1/04/01	1/05/01	1/05/01
449E	Fried Tortilla Chips	12/06/00	1/02/01	1/04/01	1/05/01	1/05/01
449F	Baked Taco Shells	12/06/00	1/02/01	1/04/01	1/05/01	1/05/01
450D	Corn Puffs	12/07/00	1/02/01	1/4/2001; 1/25/2001	1/5/2001; 1/25/2001	1/5/2001; 1/25/2001
452B	Corn Puffs	12/12/00	1/02/01	1/4/2001; 1/25/2001	1/5/2001; 1/25/2001	1/5/2001; 1/25/2001
450C	Puffed cereal (FRI)	12/07/00	1/02/01	1/04/01	1/05/01	1/05/01
453B	Corn Flakes	12/20/00	1/02/01	1/4/2001; 1/25/2001	1/5/2001; 1/25/2001	1/5/2001; 1/25/2001
456C	Polenta	1/09/01	NA	1/11/2001; 1/25/2001	1/12/2001; 1/25/2001	1/12/2001; 1/25/2001
456D	Polenta	1/09/01	NA	1/11/2001; 1/25/2001	1/12/2001; 1/25/2001; 1/26/2001	1/12/2001; 1/25/2001
456H	Hush Puppies	1/09/01	1/10/01	1/11/01	1/12/01	1/12/01
456I	Corn Muffins	1/09/01	1/10/01	1/11/01	1/12/01	1/12/01
456J	Corn Muffins	1/09/01	1/10/01	1/11/2001; 1/25/2001	1/12/2001; 1/25/2001	1/12/2001; 1/25/2001
456M	Corn Bread	1/10/01	NA	1/11/2001; 1/25/2001	1/12/2001; 1/25/2001	1/12/2001; 1/25/2001
456N	Corn Bread	1/10/01	NA	1/11/2001; 1/25/2001	1/12/2001; 1/25/2001	1/12/2001; 1/25/2001

Appendix 3: ELISA and TEP raw data

Table A3-1: Levels of Cry9C protein in processed foods produced from non-StarLink grain – Envirologix ELISA summary data

Field Sample ID: 04, Control Grain		Sample I (ppb result)		Sample II (ppb result)		Cry9C (ppb)	Sample I (actual ppb)*		Sample II (actual ppb)*		Cry9C (actual ppb)		
Sample ID:	Matrix	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	SD	% CV
418D	Masa (dough)	-0.093	-0.098	-0.100	-0.098	-0.097	-0.465	-0.490	-0.500	-0.490	-0.486	0.015	-3.07
451A	Soft Tortillas	-0.177	-0.168	-0.179	-0.173	-0.174	-0.885	-0.840	-0.895	-0.865	-0.871	0.024	-2.79
418N	Soft Tortillas	-0.153	-0.169	-0.164	-0.159	-0.161	-0.765	-0.845	-0.820	-0.795	-0.806	0.034	-4.25
418M	Fried Tortilla Chips	-0.147	-0.136	-0.150	-0.145	-0.145	-0.735	-0.680	-0.750	-0.725	-0.723	0.030	-4.17
449A	Fried Tortilla Chips	-0.153	-0.150	-0.157	-0.156	-0.154	-0.765	-0.750	-0.785	-0.780	-0.770	0.016	-2.05
449B	Fried Tortilla Chips	-0.158	-0.160	-0.160	-0.152	-0.158	-0.790	-0.800	-0.800	-0.760	-0.788	0.019	-2.40
449C	Baked Taco Shells	-0.161	-0.137	-0.176	-0.165	-0.160	-0.805	-0.685	-0.880	-0.825	-0.799	0.082	-10.29
450B	Corn Puffs	-0.111	-0.135	-0.117	-0.131	-0.124	-0.555	-0.675	-0.585	-0.655	-0.618	0.057	-9.20
452A	Corn Puffs	-0.156	-0.159	-0.148	-0.157	-0.155	-1.560	-1.590	-1.480	-1.570	-1.550	0.048	-3.12
450A	Puffed cereal (FRI)	-0.149	-0.143	-0.156	-0.133	-0.145	-0.745	-0.715	-0.780	-0.665	-0.726	0.049	-6.71
453A	Corn Flakes	-0.100	-0.083	-0.143	-0.107	-0.108	-0.500	-0.415	-0.715	-0.535	-0.541	0.126	-23.34
456A	Polenta	-0.116	-0.120	-0.117	-0.119	-0.118	-0.580	-0.600	-0.585	-0.595	-0.590	0.009	-1.55
456B	Polenta	-0.124	-0.124	-0.124	-0.128	-0.125	-0.620	-0.620	-0.620	-0.640	-0.625	0.010	-1.60
456E	Hush Puppies	-0.127	-0.135	-0.122	-0.128	-0.128	-0.635	-0.675	-0.610	-0.640	-0.640	0.027	-4.18
456F	Corn Muffins	-0.131	-0.130	-0.135	-0.137	-0.133	-0.655	-0.650	-0.675	-0.685	-0.666	0.017	-2.48
456G	Corn Muffins	-0.110	-0.102	-0.105	-0.108	-0.106	-0.550	-0.510	-0.525	-0.540	-0.531	0.018	-3.29
456K	Corn Bread	-0.130	-0.137	-0.153	-0.150	-0.143	-0.650	-0.685	-0.765	-0.750	-0.713	0.054	-7.61
456L	Corn Bread	-0.132	-0.133	-0.137	-0.135	-0.134	-0.660	-0.665	-0.685	-0.675	-0.671	0.011	-1.65
454A	Whole Grain (RAC)	-0.110	-0.093	-0.101	-0.113	-0.104	-0.550	-0.465	-0.505	-0.565	-0.521	0.045	-8.70
459A	Dry Milled Corn Meal	0.000	-0.062	-0.028	-0.051	-0.035	0.000	-0.310	-0.140	-0.255	-0.176	0.137	-77.84
461A	Dry Milled Flour	-0.050	-0.081	-0.045	-0.068	-0.061	-0.250	-0.405	-0.225	-0.340	-0.305	0.083	-27.20
454B	Wet Milled Starch	-0.132	-0.154	-0.158	-0.169	-0.153	-0.660	-0.770	-0.790	-0.845	-0.766	0.078	-10.13
454C	Wet Milled Gluten	-0.024	-0.031	-0.019	-0.033	-0.027	-0.120	-0.155	-0.095	-0.165	-0.134	0.032	-24.11
454D	Wet Milled Hull Material	-0.077	-0.095	-0.089	-0.091	-0.088	-0.385	-0.475	-0.445	-0.455	-0.440	0.039	-8.80
454E	Steepwater Concentrate	-0.117	-0.121	-0.135	-0.127	-0.125	-0.585	-0.605	-0.675	-0.635	-0.625	0.039	-6.27
457B	Wet Milled Bleached Deodorized Oil	-0.141	-0.143	-0.138	-0.141	-0.141	-0.705	-0.715	-0.690	-0.705	-0.704	0.010	-1.46

* actual ppb is based on the dilution factor incurred during extraction

Table A3-1: Levels of Cry9C protein in processed foods produced from non-StarLink grain – Envirologix ELISA summary data (continued)

Field Sample ID: 04, Control Grain		Sample I (ppb result)		Sample II (ppb result)		Cry9C (ppb)	Sample I (actual ppb)*		Sample II (actual ppb)*		Cry9C (actual ppb)		
Sample ID:	Matrix	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	SD	% CV
457A	Wet Milled Solvent Extracted Germ	0.234	0.259	0.198	0.233	0.231	2.340	2.590	1.980	2.330	2.310	0.251	10.85
457A	Wet Milled Solvent Extracted Germ	0.410	0.403	0.393	0.374	0.395	4.100	4.030	3.930	3.740	3.950	0.156	3.96
457A	Wet Milled Solvent Extracted Germ	0.365	0.375	0.493	0.484	0.429	3.650	3.750	4.930	4.840	4.293	0.686	15.99
	Averages:					0.352					3.518	0.984	27.98

* actual ppb is based on the dilution factor incurred during extraction

Table A3-2: Levels of Cry9C protein in processed foods produced from 100% StarLink grain – Envirologix ELISA summary data

Field Sample ID: 03, StarLink Grain		Sample I (ppb result)		Sample II (ppb result)		Cry9C (ppb)	Sample I (actual ppb)*		Sample II (actual ppb)*		Cry9C (actual ppb)		
Sample ID:	Matrix	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	SD	% CV
414C	Masa Dough	25.5	24.7	25.7	25.4	25.3	127.5	123.5	128.5	127.0	126.6	2.17	1.72
414A	Soft Tortillas	5.10	4.44	4.79	4.57	4.73	25.5	22.2	24.0	22.9	23.6	1.44	6.11
451B	Soft Tortillas	-0.076	-0.087	-0.058	-0.075	-0.074	-0.380	-0.435	-0.290	-0.375	-0.370	0.060	-16.18
414B	Fried Tortilla Chips	4.28	4.40	3.80	3.72	4.05	21.4	22.0	19.0	18.6	20.3	1.70	8.40
449D	Fried Tortilla Chips	-0.151	-0.049	-0.141	-0.100	-0.110	-0.755	-0.245	-0.705	-0.500	-0.551	0.232	-42.10
449E	Fried Tortilla Chips	-0.148	-0.120	-0.116	-0.088	-0.118	-0.740	-0.600	-0.580	-0.440	-0.590	0.123	-20.80
449F	Baked Taco Shells	-0.088	-0.082	-0.088	-0.078	-0.084	-0.440	-0.410	-0.440	-0.390	-0.420	0.024	-5.83
450D	Corn Puffs	-0.085	-0.088	-0.040	-0.024	-0.059	-0.425	-0.440	-0.200	-0.120	-0.296	0.161	-54.28
452B	Corn Puffs	0.469	0.468	0.441	0.445	0.456	4.69	4.68	4.41	4.45	4.56	0.148	3.25
450C	Puffed cereal (FRI)	0.872	0.805	1.01	0.923	0.903	4.36	4.03	5.05	4.62	4.51	0.432	9.58
453B	Corn Flakes	-0.060	-0.062	-0.078	-0.075	-0.069	-0.600	-0.620	-0.780	-0.750	-0.688	0.091	-13.19
456C	Polenta	102	99.0	98.4	87.2	96.7	510	495	492	436	483	32.5	6.72
456D	Polenta	124	133	107	152	129	620	665	535	760	645	93.7	14.53
456H	Hush Puppies	478	530	538	511	514	2390	2650	2690	2555	2571	133.4	5.19
456H	Hush Puppies	589	510	528	533	540	2945	2550	2640	2665	2700	170.6	6.32
456I	Corn Muffins	55.2	49.5	59.4	62.1	56.6	276	248	297	311	283	27.5	9.71
456I	Corn Muffins	198	199	226	229	213	990	995	1130	1145	1065	84.0	7.88
456J	Corn Muffins	264	267	271	278	270	1320	1335	1355	1390	1350	30.3	2.24
456J	Corn Muffins	95.7	97.1	88.1	89.1	93	479	486	441	446	463	22.8	4.93
456M	Corn Bread	478	510	411	404	451	2390	2550	2055	2020	2254	258.5	11.47
456M	Corn Bread	490	504	486	495	494	2450	2520	2430	2475	2469	38.8	1.57
456N	Corn Bread	505	503	398	419	456	2525	2515	1990	2095	2281	279.0	12.23
456N	Corn Bread	428	474	446	463	453	2140	2370	2230	2315	2264	100.6	4.44
455A	Whole Grain (RAC)	2950	2930	2670	2870	2855	14750	14650	13350	14350	14275	639.7	4.48
459B	Dry Milled Corn Meal	2940	2970	3130	3020	3015	14700	14850	15650	15100	15075	417	2.77
461B	Dry Milled Flour	3260	3110	2910	3010	3073	16300	15550	14550	15050	15363	747	4.86

Table A3-2 (continued): Levels of Cry9C protein in processed foods produced from 100% StarLink grain – Envirologix ELISA summary data.

Field Sample ID: 03, StarLink Grain		Sample I (ppb result)		Sample II (ppb result)		Cry9C (ppb)	Sample I (actual ppb)*		Sample II (actual ppb)*		Cry9C (actual ppb)		
Sample ID:	Matrix	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	SD	% CV
455B	Wet Milled Starch	2.48	2.59	2.78	2.70	2.638	12.4	13.0	13.9	13.5	13.188	0.654	4.96
455C	Wet Milled Gluten	845	817	801	787	813	4225	4085	4005	3935	4063	124.5	3.06
455D	Wet Milled Hull Material	2520	2610	2560	2670	2590	12600	13050	12800	13350	12950	324.0	2.50
455E	Steepwater Concentrate	351	331	305	283	318	1755	1655	1525	1415	1588	148.6	9.36
457C	Wet Milled Solvent Extracted Germ	2650	2500	2600	2510	2565	26500	25000	26000	25100	25650	723.4	2.82
457D	Wet Milled Bleached Deodorized Oil	-0.150	-0.147	-0.155	-0.163	-0.154	-0.750	-0.735	-0.775	-0.815	-0.769	0.035	-4.55

* actual ppb is based on the dilution factor incurred during extraction

Table A3-3: Levels of Cry9C protein in commercial taco samples – Envirologix ELISA summary data

Commercial tacos		Sample I (ppb result)		Sample II (ppb result)		Cry9C (ppb)	Sample I (actual ppb)*		Sample II (actual ppb)*		Cry9C (actual ppb)		
Sample ID:	Matrix	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	SD	% CV
010214	Tacos	-0.035	-0.035	-0.021	-0.014	-0.026	-0.175	-0.175	-0.105	-0.070	-0.131	0.053	-40.00
010215	Tacos	0.884	0.872	0.845	0.833	0.859	4.420	4.360	4.225	4.165	4.293	0.118	2.74
010216	Tacos	0.481	0.501	0.523	0.515	0.505	2.405	2.505	2.615	2.575	2.525	0.092	3.64
010217	Tacos	0.337	0.342	0.326	0.354	0.340	1.685	1.710	1.630	1.770	1.699	0.058	3.42
010220	Tacos	0.370	0.462	0.368	0.381	0.395	1.850	2.310	1.840	1.905	1.976	0.224	11.35
010221	Tacos	-0.068	-0.071	-0.007	-0.062	-0.052	-0.340	-0.355	-0.035	-0.310	-0.260	0.151	-58.14
010225	Tacos	0.048	0.038	0.029	0.031	0.037	0.240	0.190	0.145	0.155	0.183	0.043	23.51
010229	Tacos	0.173	0.172	0.174	0.174	0.173	0.865	0.860	0.870	0.870	0.866	0.005	0.55
491A	Tacos	-0.071	-0.066	-0.072	-0.071	-0.070	-0.355	-0.330	-0.360	-0.355	-0.350	0.014	-3.87

* actual ppb is based on the dilution factor (5x) incurred during extraction

Table A3-4: Levels of Cry9C protein in processed foods and milled products produced from non-StarLink grain – Aventis ELISA method summary data

Field Sample ID: 04, Control Grain		Sample I (ng/mL extract)		Sample II (ng/mL extract)		Cry9C (ng/mL)	Sample I* (ng/g sample)		Sample II* (ng/g sample)		Cry9C (ng/g sample)		
Sample ID:	Matrix	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	SD	% CV
418D	Masa (dough)	-0.138	-0.052	-0.081	0.063	-0.052	-1.394	-0.525	-0.818	0.636	-0.525	0.854	-162.66
451A	Soft Tortillas	-0.478	-0.367	-0.347	-0.276	-0.367	-4.780	-3.670	-3.436	-2.733	-3.655	0.849	-23.24
418N	Soft Tortillas	-0.718	-0.438	-0.306	-0.407	-0.467	-7.180	-4.380	-3.091	-4.111	-4.691	1.750	-37.31
418M	Fried Tortilla Chips	-0.588	-0.428	-0.558	-0.448	-0.506	-5.822	-4.238	-5.525	-4.436	-5.005	0.785	-15.69
449A	Fried Tortilla Chips	-0.458	-0.498	-0.417	-0.608	-0.495	-4.580	-4.980	-4.129	-6.020	-4.927	0.807	-16.38
449B	Fried Tortilla Chips	-0.468	-0.428	-0.608	-0.397	-0.475	-4.588	-4.196	-6.080	-3.970	-4.709	0.949	-20.16
449C	Baked Taco Shells	-0.407	-0.438	-0.478	-0.468	-0.448	-4.111	-4.424	-4.828	-4.727	-4.523	0.324	-7.16
450B	Corn Puffs	-0.708	-0.848	-0.698	-0.828	-0.771	-7.010	-8.396	-6.980	-8.280	-7.666	0.777	-10.13
452A	Corn Puffs	-1.09	-0.986	-0.887	-0.728	-0.923	-10.900	-9.860	-8.870	-7.280	-9.228	1.540	-16.69
450A	Puffed cereal (FRI)	-0.957	-0.996	-0.907	-0.748	-0.902	-9.570	-9.960	-9.070	-7.480	-9.020	1.089	-12.08
453A	Corn Flakes	-0.897	-0.828	-0.937	-0.758	-0.855	-8.881	-8.198	-9.277	-7.505	-8.465	0.780	-9.22
456A	Polenta	-0.459	-0.459	-0.421	-0.402	-0.435	-4.636	-4.636	-4.210	-4.020	-4.376	0.311	-7.10
456B	Polenta	-0.411	-0.440	-0.563	-0.525	-0.485	-4.029	-4.314	-5.745	-5.357	-4.861	0.820	-16.87
456E	Hush Puppies	-0.582	-0.667	-0.845	-0.714	-0.702	-5.820	-6.670	-8.366	-7.069	-6.981	1.060	-15.18
456F	Corn Muffins	-0.723	-0.742	-0.506	-0.544	-0.629	-7.230	-7.420	-5.060	-5.440	-6.288	1.210	-19.25
456G	Corn Muffins	-0.440	-0.468	-0.459	-0.563	-0.483	-4.444	-4.727	-4.590	-5.630	-4.848	0.534	-11.02
456K	Corn Bread	-0.535	-0.563	-0.676	-0.620	-0.599	-5.404	-5.687	-6.828	-6.263	-6.045	0.632	-10.46
456L	Corn Bread	-0.354	-0.506	-0.667	-0.573	-0.525	-3.540	-5.060	-6.604	-5.673	-5.219	1.287	-24.66
454A	Whole Grain (RAC)	-0.704	-0.784	-0.752	-0.768	-0.752	-7.040	-7.840	-7.446	-7.604	-7.482	0.337	-4.50
454B	Wet Milled Starch	-0.714	-0.670	-0.583	-0.703	-0.668	-7.212	-6.768	-5.889	-7.101	-6.742	0.600	-8.89
454C	Wet Milled Gluten	-0.880	-1.01	-0.944	-0.848	-0.921	-8.713	-10.000	-9.347	-8.396	-9.114	0.711	-7.80
454D	Wet Milled Hull Material	-0.896	-0.429	-0.720	-0.510	-0.639	-8.960	-4.290	-7.200	-5.100	-6.388	2.108	-33.01
454E	Steepwater Concentrate	-1.10	-0.704	-0.880	-0.655	-0.835	-11.000	-7.040	-8.800	-6.550	-8.348	2.015	-24.14
457A	Wet Milled Solvent Extracted Germ	-0.364	-0.411	-0.383	-0.459	-0.404	-3.604	-4.069	-3.792	-4.545	-4.002	0.409	-10.21
457B	Wet Milled Bleached Deodorized Oil	-0.468	-0.354	-0.421	-0.278	-0.380	-4.727	-3.576	-4.296	-2.837	-3.859	0.831	-21.53
459A	Dry Milled Corn Meal	-0.335	-0.458	-0.307	-0.389	-0.372	-3.418	-4.673	-3.133	-3.969	-3.798	0.679	-17.87
461A	Dry Milled Flour	-0.403	-0.594	-0.389	-0.512	-0.475	-4.071	-6.000	-3.929	-5.172	-4.793	0.978	-20.40

* ng/g sample is based on the dilution factor incurred during extraction

Table A3-5: Levels of Cry9C protein in processed foods produced from 100% StarLink grain – Aventis ELISA method summary data

Field Sample ID: 03, StarLink Grain		Sample I (ng/mL extract)		Sample II (ng/mL extract)		Cry9C (ng/mL)	Sample I* (ng/g sample)		Sample II* (ng/g sample)		Cry9C (ng/g sample)		
Sample ID:	Matrix	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	Cry9C a	Cry9C b	Cry9C a	Cry9C b	Average	SD	% CV
414C	Masa (dough)	5.11	5.78	5.48	4.16	5.13	51.62	58.38	55.35	42.02	51.84	7.11	13.71
414A	Soft Tortillas	0.586	0.530	0.813	0.685	0.654	5.860	5.300	8.130	6.850	6.535	1.24	19.00
451B	Soft Tortillas	-0.505	-0.464	-0.599	-0.532	-0.525	-5.101	-4.687	-5.990	-5.320	-5.274	0.544	-10.32
414B	Fried Tortilla Chips	-0.100	-0.100	0.000	0.045	-0.039	-1.010	-1.010	0.000	0.446	-0.394	0.735	-186.62
449D	Fried Tortilla Chips	-0.383	-0.761	-0.464	-0.532	-0.535	-3.792	-7.535	-4.687	-5.374	-5.347	1.60	-29.85
449E	Fried Tortilla Chips	-0.464	-0.613	-0.640	-0.761	-0.620	-4.687	-6.192	-6.275	-7.461	-6.154	1.14	-18.47
449F	Baked Taco Shells	-0.761	-0.814	-0.599	-0.572	-0.687	-7.610	-8.140	-5.990	-5.720	-6.865	1.19	-17.35
450D	Corn Puffs	-0.984	-1.02	-0.994	-0.951	-0.987	-9.939	-10.303	-10.143	-9.704	-10.022	0.259	-2.59
452B	Corn Puffs	-0.177	-0.022	-0.232	-0.210	-0.160	-1.806	-0.224	-2.367	-2.143	-1.635	0.968	-59.22
450C	Puffed cereal (FRI)	1.39	1.67	1.11	1.37	1.39	13.90	16.70	11.21	13.84	13.91	2.24	16.11
453B	Corn Flakes	-0.844	-0.800	-0.865	-0.865	-0.844	-8.440	-8.000	-8.737	-8.737	-8.479	0.349	-4.11
456C	Polenta	21.1	20.0	26.0	20.3	21.9	206.9	196.1	265.3	207.1	218.8	31.4	14.35
456D	Polenta	35.4	33.2	25.8	27.8	30.6	347.1	325.5	258.0	278.0	302.1	41.2	13.64
456H	Hush Puppies	113	111	126	120	118	1130	1110	1235	1176	1163	55.7	4.79
456I	Corn Muffins	8.64	7.78	8.16	6.16	7.69	85.54	77.03	81.60	61.60	76.44	10.5	13.72
456J	Corn Muffins	27.7	26.5	26.2	29.6	27.5	277.0	265.0	262.0	296.0	275.0	15.4	5.61
456M	Corn Bread	32.2	40.5	27.0	30.0	32.4	318.8	401.0	270.0	300.0	322.5	56.1	17.39
456N	Corn Bread	139	140	105	124	127	1376	1386	1040	1228	1257	162	12.91
455A	Whole Grain (RAC)	1010	1010	847	810	919	10000	10000	8556	8182	9184	954	10.39
455B	Wet Milled Starch	2.31	2.35	2.59	2.69	2.49	22.65	23.04	25.39	26.37	24.36	1.81	7.42
455C	Wet Milled Gluten	131	116	130	162	135	1323	1172	1300	1620	1354	190	14.01
455D	Wet Milled Hull Material	929	1000	926	1040	974	9290	10000	9260	10400	9738	559	5.74
455E	Steepwater Concentrate	138	110	126	105	120	1408	1122	1248	1040	1204	160	13.32
457C	Wet Milled Solvent Extracted Germ	1270	1310	1080	964	1156	12574	12970	10800	9640	11496	1556	13.54
457D	Wet Milled Bleached Deodorized Oil	-0.757	-0.724	-0.703	-0.703	-0.722	-7.422	-7.098	-7.030	-7.030	-7.145	0.187	-2.62
459B	Dry Milled Corn Meal	717	705	597	548	642	7029	6912	5970	5480	6348	748	11.78
461B	Dry Milled Flour	784	868	630	719	750	8000	8857	6364	7263	7621	1062	13.93

ng/g sample is based on the dilution factor incurred during extraction

The following tables show the data for determination of the total extractable protein found in the extracts. The TEP was determined for each sample extract in order to show that protein was being extracted from the samples during the extraction process.

The Bradford assay[1] was used to determine the concentration of total extractable protein (TEP). The assay relies on the binding of the dye Coomassie blue G250 to protein. The anionic form of the dye, which binds to protein, has a maximum absorption at 595 nm. The amount of absorption at 595 nm produced is therefore correlated to the protein concentration. Bovine Serum Albumin (BSA) was used as protein standard at 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL in the assay.

The total extractable protein was determined for each sample extract. Duplicate 10 µL aliquots of the sample extract were placed in wells of a 96-well plate (Costar No. 3590) and 200 µL of Bradford Reagent (Sigma) was added. After 12 ± 3 minutes of incubation on a shaker (IKA-SCHÜTTLER MTS 4) at 700 rpm at room temperature, the optical density (OD) was measured in a microplate reader (Molecular Devices THERMOmax) at 595 nm.

REFERENCES:

1. Bradford, M.M. “A refined and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.” Anal. Biochem., 72, 248 (1976).

Table A3-6: Levels of total extractable protein (TEP) in extracts of processed foods produced from non-StarLink grain – Envirologix method data

Field Sample ID: 04, Control Grain		Sample I (mg/mL extract)		Sample II (mg/mL extract)		Sample I (mg/g sample)		Sample II (mg/g sample)		TEP (mg/g sample)		
Sample ID:	Matrix	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	Average	SD	% CV
418D	Masa (dough)	0.093	0.090	0.107	0.105	0.47	0.45	0.54	0.53	0.50	0.04	8.61
451A	Soft Tortillas	0.196	0.181	0.189	0.186	0.97	0.90	0.95	0.93	0.94	0.03	3.32
418N	Soft Tortillas	0.189	0.201	0.171	0.188	0.94	1.00	0.84	0.92	0.92	0.06	7.01
418M	Fried Tortilla Chips	0.168	0.172	0.174	0.166	0.83	0.85	0.88	0.84	0.85	0.02	2.45
449A	Fried Tortilla Chips	0.223	0.219	0.241	0.239	1.13	1.11	1.22	1.21	1.16	0.06	4.82
449B	Fried Tortilla Chips	0.241	0.235	0.236	0.234	1.21	1.18	1.18	1.17	1.18	0.02	1.31
449C	Baked Taco Shells	0.370	0.331	0.355	0.328	1.85	1.66	1.79	1.66	1.74	0.10	5.66
450B	Corn Puffs	0.209	0.289	0.263	0.317	1.05	1.45	1.30	1.57	1.34	0.23	16.80
452A	Corn Puffs	0.150	0.150	0.166	0.160	1.53	1.53	1.69	1.63	1.60	0.08	5.04
450A	Puffed cereal (FRI)	0.335	0.361	0.244	0.356	0.83	0.90	0.61	0.89	0.81	0.13	16.71
453A	Corn Flakes	0.162	0.183	0.151	0.183	0.41	0.46	0.38	0.46	0.42	0.04	9.51
456A	Polenta	1.85	1.77	2.15	2.21	9.25	8.85	10.64	10.94	9.92	1.03	10.34
456B	Polenta	1.45	1.53	1.54	1.58	7.32	7.73	7.78	7.98	7.70	0.28	3.57
456E	Hush Puppies	1.16	1.22	1.24	1.31	5.74	6.04	6.14	6.49	6.10	0.31	5.02
456F	Corn Muffins	1.11	1.17	0.976	1.14	5.50	5.79	4.93	5.76	5.49	0.40	7.26
456G	Corn Muffins	1.10	1.12	1.10	1.13	5.45	5.54	5.56	5.71	5.56	0.11	1.94
456K	Corn Bread	2.03	2.03	1.54	1.47	10.25	10.25	7.70	7.35	8.89	1.58	17.79
456L	Corn Bread	1.96	2.07	1.67	1.71	9.70	10.25	8.35	8.55	9.21	0.91	9.90
454A	Whole Grain (RAC)	2.55	2.34	1.93	1.79	12.62	11.58	9.65	8.95	10.70	1.70	15.86
459A	Dry Milled Corn Meal	2.35	2.32	2.40	2.37	11.99	11.84	11.88	11.73	11.86	0.11	0.90
461A	Dry Milled Flour	2.85	2.72	2.85	2.61	13.97	13.33	14.39	13.18	13.72	0.56	4.11
454B	Wet Milled Starch	0.035	0.029	0.034	0.031	0.18	0.15	0.17	0.15	0.16	0.01	8.52
454C	Wet Milled Gluten	0.554	0.543	0.558	0.569	2.80	2.74	2.76	2.82	2.78	0.03	1.21
454D	Wet Milled Hull Material	0.641	0.744	0.607	0.770	3.17	3.68	3.04	3.85	3.44	0.39	11.43
454E	Steepwater Concentrate	0.049	0.050	0.045	0.043	0.24	0.25	0.23	0.22	0.23	0.02	6.52
457B	Wet Milled Bleached Deodorized Oil	0.038	0.035	0.034	0.035	0.19	0.17	0.17	0.17	0.17	0.01	5.28

Table A3-6: Levels of total extractable protein (TEP) in extracts of processed foods produced from non-StarLink grain – Envirologix method data (continued)

Field Sample ID: 04, Control Grain		Sample I (mg/mL extract)		Sample II (mg/mL extract)		Sample I (mg/g sample)		Sample II (mg/g sample)		TEP (mg/g sample)		
Sample ID:	Matrix	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	Average	SD	% CV
457A	Wet Milled Solvent Extracted Germ	5.98	5.50	5.88	5.46	59.80	55.00	58.80	54.60	57.05	2.64	4.62
457A	Wet Milled Solvent Extracted Germ	6.93	6.56	6.99	6.56	67.94	64.31	68.53	64.31	66.27	2.28	3.44
457A	Wet Milled Solvent Extracted Germ	9.16	9.12	7.95	7.98	45.35	45.15	39.55	39.70	42.44	3.25	7.65
	Combined data for for Wet Milled Solvent Extracted Germ									55.25	10.55	19.09

Table A3-7: Levels of TEP in extracts of processed foods produced from 100% StarLink grain – Envirologix method data

Field Sample ID: 03, StarLink Grain		Sample I (mg/mL extract)		Sample II (mg/mL extract)		Sample I (mg/g sample)		Sample II (mg/g sample)		TEP (mg/g sample)		
Sample ID:	Matrix	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	Average	SD	% CV
414C	Masa (dough)	0.153	0.147	0.161	0.159	0.750	0.721	0.789	0.779	0.760	0.03	4.08
414A	Soft Tortillas	0.195	0.211	0.215	0.246	0.98	1.07	1.09	1.24	1.09	0.11	9.84
451B	Soft Tortillas	0.193	0.251	0.194	0.235	0.98	1.28	0.99	1.20	1.11	0.15	13.43
414B	Fried Tortilla Chips	0.310	0.350	0.340	0.325	1.55	1.75	1.72	1.64	1.66	0.09	5.34
449D	Fried Tortilla Chips	0.411	0.335	0.404	0.344	2.03	1.66	2.04	1.74	1.87	0.20	10.64
449E	Fried Tortilla Chips	0.450	0.326	0.403	0.302	2.27	1.65	2.00	1.50	1.85	0.35	18.89
449F	Baked Taco Shells	0.295	0.301	0.291	0.279	1.48	1.51	1.44	1.38	1.45	0.05	3.66
450D	Corn Puffs	0.263	0.289	0.274	0.283	1.33	1.46	1.38	1.43	1.40	0.06	4.08
452B	Corn Puffs	0.165	0.168	0.154	0.157	1.63	1.66	1.54	1.57	1.60	0.06	3.54
450C	Puffed cereal (FRI)	0.370	0.411	0.301	0.347	1.87	2.08	1.52	1.75	1.80	0.23	12.85
453B	Corn Flakes	0.053	0.046	0.053	0.051	0.52	0.46	0.54	0.52	0.51	0.04	7.05
456C	Polenta	1.79	1.83	1.92	1.97	8.86	9.06	9.50	9.75	9.29	0.41	4.38
456D	Polenta	1.35	1.34	1.27	1.23	6.89	6.84	6.41	6.21	6.59	0.33	4.98
456H	Hush Puppies	1.33	1.27	1.16	1.15	6.52	6.23	5.80	5.75	6.07	0.37	6.02
456H	Hush Puppies	1.88	2.25	2.01	2.17	9.40	11.25	10.05	10.85	10.39	0.83	7.95
456I	Corn Muffins	1.16	1.16	1.16	1.10	5.86	5.86	5.74	5.45	5.73	0.20	3.41
456I	Corn Muffins	0.91	0.87	0.98	0.88	4.57	4.39	4.79	4.30	4.52	0.22	4.79
456J	Corn Muffins	1.26	1.38	1.32	1.42	6.36	6.97	6.60	7.10	6.76	0.34	5.00
456J	Corn Muffins	0.74	0.72	0.76	0.73	3.68	3.55	3.82	3.68	3.68	0.11	2.98
456M	Corn Bread	2.35	2.41	1.94	1.80	11.87	12.17	9.70	9.00	10.69	1.57	14.72
456M	Corn Bread	1.10	1.11	1.06	1.06	5.45	5.50	5.30	5.30	5.39	0.10	1.86
456N	Corn Bread	1.93	2.02	1.55	1.55	9.65	10.10	7.67	7.67	8.77	1.28	14.64
456N	Corn Bread	0.97	1.01	1.02	1.03	4.82	5.00	5.05	5.10	4.99	0.12	2.47
455A	Whole Grain (RAC)	2.33	2.44	2.71	2.64	11.53	12.08	13.69	13.33	12.66	1.02	8.05
455B	Wet Milled Starch	0.022	0.020	0.020	0.019	0.11	0.10	0.10	0.09	0.10	0.01	7.07
455C	Wet Milled Gluten	0.554	0.498	0.589	0.600	2.77	2.49	2.95	3.00	2.80	0.23	8.19
455D	Wet Milled Hull Material	1.31	1.32	1.34	1.33	6.55	6.60	6.70	6.65	6.63	0.06	0.97
455E	Steepwater Concentrate	0.114	0.103	0.102	0.098	0.57	0.52	0.52	0.49	0.52	0.03	6.16
457C	Wet Milled Solvent Extracted Germ	5.25	5.19	5.59	5.42	52.50	51.90	55.35	53.66	53.35	1.52	2.84
457D	Wet Milled Bleached Deodorized Oil	0.022	0.023	0.028	0.032	0.11	0.12	0.14	0.16	0.13	0.02	16.63
459B	Dry Milled Corn Meal	0.899	0.876	0.793	0.768	8.81	8.59	7.93	7.68	8.25	0.54	6.49
461B	Dry Milled Flour	0.781	0.739	0.756	0.717	7.97	7.54	7.64	7.24	7.60	0.30	3.94

Table A3-8: Levels of TEP in extracts of processed foods produced from non-StarLink grain – Aventis method data

Field Sample ID: 04, Control Grain		Sample I (mg/mL extract)		Sample II (mg/mL extract)		Sample I (mg/g sample)		Sample II (mg/g sample)		TEP (mg/g sample)		
Sample ID:	Matrix	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	Average	SD	% CV
418D	Masa (dough)	0.043	0.042	0.042	0.043	0.434	0.424	0.424	0.434	0.429	0.01	1.36
451A	Soft Tortillas	0.049	0.044	0.044	0.042	0.49	0.44	0.44	0.42	0.45	0.03	7.09
418N	Soft Tortillas	0.030	0.034	0.034	0.034	0.30	0.34	0.34	0.34	0.33	0.02	6.39
418M	Fried Tortilla Chips	0.036	0.036	0.029	0.035	0.36	0.36	0.29	0.35	0.34	0.03	9.90
449A	Fried Tortilla Chips	0.046	0.052	0.051	0.060	0.46	0.52	0.50	0.59	0.52	0.06	10.72
449B	Fried Tortilla Chips	0.061	0.055	0.058	0.052	0.60	0.54	0.58	0.52	0.56	0.04	6.43
449C	Baked Taco Shells	0.063	0.061	0.060	0.063	0.64	0.62	0.61	0.64	0.62	0.02	2.43
450B	Corn Puffs	0.069	0.067	0.075	0.066	0.68	0.66	0.75	0.66	0.69	0.04	6.07
452A	Corn Puffs	0.073	0.073	0.067	0.067	0.73	0.73	0.67	0.67	0.70	0.03	4.95
450A	Puffed cereal (FRI)	0.078	0.082	0.078	0.080	0.78	0.82	0.78	0.80	0.80	0.02	2.41
453A	Corn Flakes	0.040	0.037	0.038	0.038	0.40	0.37	0.38	0.38	0.38	0.01	3.29
456A	Polenta	0.271	0.278	0.277	0.286	2.74	2.81	2.77	2.86	2.79	0.05	1.89
456B	Polenta	0.202	0.197	0.210	0.216	1.98	1.93	2.14	2.20	2.06	0.13	6.28
456E	Hush Puppies	0.218	0.222	0.245	0.246	2.18	2.22	2.43	2.44	2.32	0.13	5.80
456F	Corn Muffins	0.235	0.240	0.228	0.243	2.35	2.40	2.28	2.43	2.37	0.07	2.77
456G	Corn Muffins	0.232	0.208	0.258	0.268	2.34	2.10	2.58	2.68	2.43	0.26	10.66
456K	Corn Bread	0.327	0.329	0.433	0.425	3.30	3.32	4.37	4.29	3.82	0.59	15.43
456L	Corn Bread	0.491	0.503	0.404	0.381	4.91	5.03	4.00	3.77	4.43	0.63	14.33
454A	Whole Grain (RAC)	0.474	0.482	0.566	0.584	4.74	4.82	5.60	5.78	5.24	0.53	10.18
459A	Dry Milled Corn Meal	0.350	0.338	0.332	0.331	3.57	3.45	3.39	3.38	3.45	0.09	2.59
461A	Dry Milled Flour	0.459	0.464	0.493	0.475	4.64	4.69	4.98	4.80	4.78	0.15	3.19
454B	Wet Milled Starch	0.028	0.030	0.030	0.029	0.28	0.30	0.30	0.29	0.30	0.01	3.27
454C	Wet Milled Gluten	0.133	0.133	0.121	0.122	1.32	1.32	1.20	1.21	1.26	0.07	5.23
454D	Wet Milled Hull Material	0.122	0.127	0.122	0.133	1.22	1.27	1.22	1.33	1.26	0.05	4.15
454E	Steepwater Concentrate	0.029	0.030	0.031	0.034	0.29	0.30	0.31	0.34	0.31	0.02	6.97
457A	Wet Milled Solvent Extracted Germ	0.175	0.178	0.200	0.184	1.73	1.76	1.98	1.82	1.82	0.11	6.05
457B	Wet Milled Bleached Deodorized Oil	0.015	0.011	0.014	0.010	0.15	0.11	0.14	0.10	0.13	0.02	18.91

Table A3-9: Levels of TEP in extracts of processed foods produced from 100% StarLink grain – Aventis method data

Field Sample ID: 03, StarLink Grain		Sample I (mg/mL extract)		Sample II (mg/mL extract)		Sample I (mg/g sample)		Sample II (mg/g sample)		TEP (mg/g sample)		
Sample ID:	Matrix	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	TEP a	TEP b	Average	SD	% CV
414C	Masa Dough	0.153	0.147	0.161	0.159	0.750	0.721	0.789	0.779	0.760	0.03	4.08
414A	Soft Tortillas	0.044	0.042	0.049	0.047	0.44	0.42	0.49	0.47	0.46	0.03	6.83
451B	Soft Tortillas	0.036	0.033	0.038	0.041	0.36	0.33	0.38	0.41	0.37	0.03	8.61
414B	Fried Tortilla Chips	0.057	0.059	0.06	0.062	0.58	0.60	0.59	0.61	0.59	0.02	2.62
449D	Fried Tortilla Chips	0.043	0.050	0.034	0.040	0.43	0.50	0.34	0.40	0.42	0.06	15.00
449E	Fried Tortilla Chips	0.034	0.042	0.035	0.041	0.34	0.42	0.34	0.40	0.38	0.04	10.93
449F	Baked Taco Shells	0.071	0.067	0.071	0.067	0.71	0.67	0.71	0.67	0.69	0.02	3.35
450D	Corn Puffs	0.161	0.167	0.146	0.152	1.63	1.69	1.49	1.55	1.59	0.09	5.42
452B	Corn Puffs	0.098	0.097	0.087	0.087	1.00	0.99	0.89	0.89	0.94	0.06	6.59
450C	Puffed cereal (FRI)	0.113	0.100	0.130	0.117	1.13	1.00	1.31	1.18	1.16	0.13	11.21
453B	Corn Flakes	0.039	0.039	0.038	0.040	0.39	0.39	0.38	0.40	0.39	0.01	2.18
456C	Polenta	0.488	0.490	0.349	0.338	4.78	4.80	3.56	3.45	4.15	0.75	17.97
456D	Polenta	0.293	0.265	0.384	0.337	2.87	2.60	3.84	3.37	3.17	0.55	17.32
456H	Hush Puppies	0.346	0.335	0.370	0.376	3.46	3.35	3.63	3.69	3.53	0.15	4.36
456I	Corn Muffins	0.233	0.243	0.211	0.220	2.31	2.41	2.11	2.20	2.26	0.13	5.70
456J	Corn Muffins	0.430	0.490	0.392	0.411	4.30	4.90	3.92	4.11	4.31	0.42	9.85
456M	Corn Bread	0.783	0.786	0.605	0.585	7.75	7.78	6.05	5.85	6.86	1.05	15.35
456N	Corn Bread	0.998	0.998	1.12	1.09	9.88	9.88	11.09	10.79	10.41	0.62	5.99
455A	Whole Grain (RAC)	1.01	0.849	0.896	0.889	10.00	8.41	9.05	8.98	9.11	0.66	7.25
459B	Dry Milled Corn Meal	0.899	0.876	0.793	0.768	8.81	8.59	7.93	7.68	8.25	0.54	6.49
461B	Dry Milled Flour	0.781	0.739	0.756	0.717	7.97	7.54	7.64	7.24	7.60	0.30	3.94
455B	Wet Milled Starch	0.012	0.010	0.015	0.011	0.12	0.10	0.15	0.11	0.12	0.02	18.00
455C	Wet Milled Gluten	0.163	0.179	0.177	0.186	1.65	1.81	1.77	1.86	1.77	0.09	5.13
455D	Wet Milled Hull Material	0.336	0.385	0.305	0.363	3.36	3.85	3.05	3.63	3.47	0.35	9.95
455E	Steepwater Concentrate	0.080	0.073	0.079	0.069	0.82	0.74	0.78	0.68	0.76	0.06	7.53
457C	Wet Milled Solvent Extracted Germ	0.167	0.174	0.156	0.154	1.65	1.72	1.56	1.54	1.62	0.09	5.25
457D	Wet Milled Bleached Deodorized Oil	0.025	0.023	0.023	0.023	0.25	0.23	0.23	0.23	0.23	0.01	3.68

Appendix 4: Validation of EnviroLogix ELISA.

Reference Substances

Cry9C protein
Chemical name: Insecticidal Crystal Protein 9C
Molecular Weight: 70 kDa

Cry9C protein reference substances and antibodies specifically recognizing each target protein were supplied by Aventis CropScience Belgium SA (Gent, Belgium). Upon arrival at Aventis CropScience USA (formerly AgrEvo USA Company), each component was assigned a unique lot number. Cry9C reference substance was also used to fortify non-StarLink samples for validation and recovery studies.

Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined for the EnviroLogix ELISA method for each matrix type produced in this study using samples produced from non-StarLink grain. Samples were spiked with the Cry9C standard obtained from Aventis CropScience Belgium SA (Gent, Belgium).

The purpose of the validation was to determine the limit of quantitation of the method for each matrix. Thus Non-StarLink control samples of each matrix were each fortified with 0.2, 0.5, 1 and 3ng/mL Cry9C in the extraction buffer (equivalent to 10, 25, 50 and 150 ng Cry9C /g fresh weight in the matrix) prior to extraction. Five replicates extractions were prepared, and each replicate was analyzed using duplicate wells.

An LOD was determined from examination of the data for the non-StarLink samples. The LOD was determined for each matrix using the average standard curve and the concentration derived from the background optical density (OD) of the negative control samples. All the non-StarLink samples showed a small negative value on the ELISA assay, which is indicative that they contained no Cry9C protein.

The limit of quantitation (LOQ) is defined as the lowest concentration of the standard that meets the criteria for the LOQ. Validity criteria are (a) analyte recoveries from fortified matrix samples are $\geq 60\%$ and $\leq 130\%$ and (b) the coefficient of variance (relative standard deviation) is less than 25%. When a lower recovery is caused by the nature of a specific matrix or by the effect of the matrix, the lowest concentration of the standard that gives a smaller coefficient of variance than 25% is used as the LOQ. In two matrices, tortilla chips and baked taco shells, the recovery was greater than 130% at all levels. In these cases, the lowest level giving a CV of less than 25% was taken as the LOQ.

An ELISA reading giving rise to a Cry9C protein concentration below the LOD is assumed to be equal to the zero dose reading and is reported as ND (Non-detectable). Values below the LOQ but above the LOD are reported as '<LOQ'. The limits of detection and limits of quantification are summarized in Table A4-1.

Table A4-1: LOD and LOQ for matrices used in this study.

Process	BT-ID	Commodity	LOD [a] (ng/mL)	LOD (ng/g) [b]	LOQ (ng/mL)	LOQ (ng/g)
	454A	Whole Corn	0.07	0.35	0.5	2.5
Dry Mill	459A	Meal	0.07	0.35	0.5	2.5
	461A	Flour	0.07	0.35	0.5	2.5
Wet Mill	454B	Starch	0.07	0.35	0.2	1
	454C	Gluten	0.07	0.35	0.5	2.5
	457A	Solvent extracted Germ	0.65	6.42	3.0	30
	457B, 237O	Refined Oil	0.07	0.35	0.2	1
Processed Foods	418M	Tortilla Chips	0.07	0.35	0.2	1
	449A	Soft Tortillas	0.07	0.35	0.2	1
	449C	Baked Taco Shells	0.07	0.35	1	5
	450A	Puffed Cereal	0.07	0.35	0.2	1
	452A	Corn Puffs	0.07	0.35	0.5	2.5
	453A	Corn Flakes	0.07	0.35	0.2	1
	456E	Hush Puppies	0.07	0.35	0.5	2.5
	456F	Corn Muffins	0.07	0.35	0.5	2.5
	456K	Corn Bread	0.07	0.35	0.5	2.5
	456B	Polenta	0.07	0.35	0.5	2.5
	418D	Masa (dough)	0.07	0.35	0.2	1
	491A	Commercial Tacos	0.07	0.35	0.5	2.5

[a] LOD (Limit of Detection) - based on the manufacturer's specification, or data generated from non-StarLink samples in this study, whichever was the higher.

[b] The LOQ, expressed in ng/g, i.e. ppb, was calculated based on the extraction of 1 g matrix/5 mL (or 10mL as appropriate) extraction buffer (EnviroLogix method) .

[c] Commercial Tacos gave elevated values for recovery (>130%). The LOQ shown is based on a calculated recovery of 150%.

Critical dates are given in Table A4-2. A summary of the recovery data and descriptive statistics of the data are shown in Tables A4-3 through A4_22).

Table A4-2: Critical dates for method validation in processed foods – Envirologix method

Biotech ID #:	Matrix	Sample received	Sample ground	Sample extracted	Cry9C assayed
454A	Whole Grain	1/3/01	1/4/01	1/31/01	2/1/01
459A	Corn Meal	1/19/01	NA	1/31/01	2/1/01
461A	Corn Flour	1/26/01	NA	2/1/01	2/2/01
454B	Starch	1/3/01	NA	2/1/01	2/2/01
454C	Gluten	1/3/01	NA	2/8/01	2/9/01
457A	Solvent extracted Germ	1/10/01	NA	3/27/01	3/28/01
457B	Refined Oil	1/10/01	NA	2/8/01	2/9/01
418M	Tortilla Chips (not reported)*	11/17/00	1/2/01, 2/2/01	2/11/01	2/12/01
449A	Tortilla Chips*	12/6/00	2/01/01	3/29/01	3/30/01
418N	Soft Tortillas*	11/17/00	11/17/00	2/11/00, 3/29/01	2/12/00, 3/30/01
449C	Baked Taco Shells	12/6/00	1/2/01	#	#
237O	Refined Oil	3/4/99	NA	2/12/01	2/13/01
450A	Puffed Cereal	12/7/00	2/2/01	2/12/01	2/13/01
452A	Corn Puffs	12/12/00	2/13/01	2/13/01	2/14/01
453A	Corn Flakes	12/20/00	1/2/01	2/13/01	2/14/01
456E	Hush Puppies	1/9/01	2/2/01	2/13/01	2/14/01
456F	Corn Muffins	1/9/01	2/2/01	2/13/01	2/14/01
456K	Corn Bread	1/10/01	NA	2/19/01	2/20/01
456B	Polenta	1/9/01	NA	2/19/01	2/20/01
418D	Masa (dough)	11/16/00	NA	2/21/01	2/22/01
491A	Commercial Taco Chips	3/19/01	3/19/01	3/20/01	3/21/01

* Validations were repeated because of suspicion that the wrong ground sample had been taken for validation on 2/11 and 2/12/01. The validation data from 3/30/01 was used in this study.
# Raw data misplaced..

Validation Tables: The % recoveries of Cry9C protein are the averages of 5 extract replicates per fortification level with duplicate ELISA analysis of each extract (10 data points per fortification level). The standard deviation (SD) and Coefficient of Variation (%CV) are also shown.

Table A4-3: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Whole Grain.

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate:	1	2	3	4	5	6	7	8	9	10			
454A	3	15	68	59.3	69.7	68.3	66.7	64.7	59.7	71.7	62.0	63.7	65.4	4.2	6.4
454A	1	5	85.7	82.4	87.5	84.7	89.3	87.1	90	85	82.6	84.9	85.9	2.6	3.0
454A	0.5	2.5	65.8	74.6	70.6	70.4	64	61.6	65.4	63	65.4	66	66.7	4.0	6.0
454A	0.2	1	155.5	148	169.5	160.5	161.5	148	154.5	162.5	149	147	155.6	7.7	5.0

Table A4-4: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Corn Meal.

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
459A	3	15	71.3	64.7	71.3	65.3	65.3	65.3	65.0	65.7	65.3	64.7	66.4	2.6	3.9
459A	1	5	89.5	87.5	86.0	86.1	86.8	88.5	99.4	89.3	93.9	92.0	89.9	4.2	4.7
459A	0.5	2.5	83.0	90.8	83.6	72.2	85.6	87.0	77.6	79.2	72.8	72.8	80.5	6.6	8.2
459A	0.2	1	209.5	196	201.5	183.5	186.5	194	195.5	192	198	196.5	195.3	7.3	3.7

Table A4-5: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Flour.

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
461A	3.0	15.0	70.0	64.0	70.0	67.3	69.3	68.0	70.0	67.0	69.3	65.7	68.1	2.1	3.0
461A	1.0	5.0	73.4	67.7	74.7	66.4	89.5	69.5	68.9	65.3	68.1	77.4	72.1	7.2	10.0
461A	0.5	2.5	135.8	135.8	141.4	123.2	122.4	120.6	137.0	116.6	129.8	111.4	127.4	10.0	7.8
461A	0.2	1.0	145.0	123.0	151.0	129.0	136.0	130.0	143.5	127.5	134.0	157.0	137.6	11.1	8.1

Table A4-6: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Starch.

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
454B	3	15	49.7	49.0	47.7	46.3	50.7	52.3	47.0	49.7	48.3	50.0	49.1	1.8	3.7
454B	1	5	33.1	33.4	35.4	32.7	39.0	31.9	33.9	37.2	39.9	38.6	35.5	2.9	8.3
454B	0.5	2.5	56.4	38.8	48.8	48.8	49.0	47.2	54.0	49.6	49.2	49.2	49.1	4.6	9.3
454B	0.2	1	44.5	49.0	56.0	61.0	54.5	53.5	49.5	47.5	54.5	60.0	53.0	5.3	10.1

Table A4-7: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Gluten.

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
454C	3	15	133.3	132.7	152.7	153.3	141.3	140.0	147.7	142.7	134.3	131.7	141.0	8.1	5.8
454C	1	5	135.0	135.0	138.0	133.0	145.0	129.0	131.0	133.0	136.0	136.0	135.1	4.4	3.2
454C	0.5	2.5	111.2	116.8	108.8	107.4	113.6	108.0	108.4	130.2	106.0	107.0	111.7	7.3	6.5
454C	0.2	1	128.5	135.0	125.0	156.0	123.5	195.0	142.5	142.0	135.0	131.5	141.4	21.1	15.0

Table A4-8: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Refined Oil.

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
457B	3	15	97.0	103.3	92.7	101.7	95.3	94.7	101.0	113.7	100.0	92.7	99.2	6.3	6.4
457B	1	5	124.0	116.0	118.0	107.0	115.0	122.0	119.0	114.0	117.0	115.0	116.7	4.7	4.0
457B	0.5	2.5	99.6	89.8	96.4	91.2	83.2	82.0	78.4	78.8	72.6	75.6	84.8	9.1	10.7
457B	0.2	1	73.5	88.5	90.0	74.5	101.0	104.0	63.5	65.5	69.5	68.0	79.8	14.9	18.7

Table A4-9: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Soft Tortillas.

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
418N	3	15	108.00	111.67	105.33	106.33	103.67	107.00	102.67	105.00	101.00	107.67	105.83	3.03	2.86
418N	1	5	109.00	109.00	106.00	105.00	103.00	105.00	106.00	107.00	102.00	102.00	105.40	2.55	2.42
418N	0.5	2.5	117.80	112.00	114.20	107.20	109.80	107.60	109.00	111.00	106.00	103.00	109.76	4.25	3.87
418N	0.2	1	91.50	90.00	98.50	86.50	90.00	87.50	91.50	90.50	91.50	92.00	90.95	3.21	3.53

Table A4-10: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Tortilla Chips.

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
449A	3	15	142.33	145.00	149.67	147.67	154.00	160.67	142.33	143.33	149.00	150.00	148.40	5.75	3.88
449A	1	5	135.00	139.00	138.00	138.00	144.00	141.00	135.00	140.00	140.00	134.00	138.40	3.10	2.24
449A	0.5	2.5	167.80	161.40	163.60	157.60	168.00	171.20	161.60	164.20	169.00	174.00	165.84	5.02	3.03
449A	0.2	1	160.50	160.00	296.50	173.00	165.00	161.00	163.50	163.00	165.50	170.50	177.85	41.90	23.56

Table A4-11: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Baked Taco Shells

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
449C	3	15	95.33	94.67	109.00	97.00	101.33	100.67	123.67	158.67	95.33	93.67	106.93	20.35	19.03
449C	1	5	100.00	99.70	101.00	97.40	98.00	98.80	102.00	99.90	101.00	99.40	99.72	1.41	1.41
449C	0.5	2.5	140.80	109.60	108.40	166.60	132.00	109.60	117.40	102.00	113.00	204.00	130.34	32.43	24.88
449C	0.2	1	80.00	325.00	98.50	125.50	77.50	65.00	87.00	75.50	75.50	87.00	109.65	77.47	70.65

Table A4-12: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Refined oil (from CM98B003).

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
237O	3	15	38.7	41.3	45.0	44.7	35.7	40.0	38.0	38.3	40.3	39.7	40.2	2.9	7.2
237O	1	5	32.6	39.7	34.7	39.4	27.0	32.9	30.1	33.6	41.7	38.8	35.1	4.7	13.5
237O	0.5	2.5	29.2	44.6	44.2	46.6	38.2	55.4	42.6	51.6	50.2	56.2	45.9	8.2	17.8
237O	0.2	1	63.0	70.5	72.0	68.0	76.5	61.5	87.5	94.5	73.5	63.0	73.0	10.8	14.8

Table A4-13: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Puffed Cereal (Shukla).

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
450A	3	15	69.3	66.0	70.3	63.3	69.3	63.0	64.0	64.0	66.3	61.0	65.7	3.1	4.8
450A	1	5	46.7	48.5	49.9	53.6	46.2	47.2	47.2	46.5	45.7	47.0	47.9	2.4	4.9
450A	0.5	2.5	59.0	55.8	57.0	63.0	61.0	65.8	56.4	58.0	89.2	90.4	65.6	13.1	20.1
450A	0.2	1	73.0	65.0	83.0	75.0	97.0	80.5	62.5	70.5	78.0	71.0	75.6	9.9	13.1

Table A4-14: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Corn Puffs (Diehl)

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
452A	3	15	37.0	40.0	41.7	43.7	43.3	38.0	47.0	48.0	38.7	35.7	41.3	4.2	10.1
452A	1	5	40.7	40.4	43.1	41.4	43.4	40.5	38.9	38.9	36.9	34.7	39.9	2.7	6.7
452A	0.5	2.5	39.8	39.8	39.2	38.4	45.4	42.4	38.2	38.8	39.2	39.6	40.1	2.2	5.5
452A	0.2	1	36.5	45.0	41.5	40.5	65.0	42.0	51.5	51.0	41.5	165.0	58.0	38.5	66.4

Table A4-15: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Corn Flakes

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
453A	3	15	94.3	99.3	87.7	89.0	92.7	93.0	85.3	83.7	93.0	89.7	90.8	4.6	5.1
453A	1	5	119.0	114.0	109.0	104.0	118.0	111.0	105.0	92.6	93.8	92.0	105.8	10.2	9.7
453A	0.5	2.5	110.6	105.8	118.0	110.8	112.6	106.8	107.4	101.8	110.0	110.8	109.5	4.4	4.0
453A	0.2	1	112.5	113.0	121.5	120.5	127.0	120.5	126.0	137.5	135.5	129.0	124.3	8.4	6.8

Table A4-16: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Hush Puppies

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
456E	3	15	54.7	54.0	53.3	52.7	54.0	52.7	52.7	53.7	54.3	52.0	53.4	0.9	1.6
456E	1	5	75.2	76.9	78.6	74.9	76.9	72.8	76.0	74.6	74.4	72.2	75.3	1.9	2.6
456E	0.5	2.5	67.8	64.6	68.0	64.2	66.4	64.2	70.4	67.0	72.8	68.6	67.4	2.8	4.1
456E	0.2	1	66.5	49.5	48.5	46.0	60.0	44.5	49.0	57.5	49.0	40.0	51.1	7.9	15.6

Table A4-17: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Muffins

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
456F	3	15	48.7	48.3	93.7	52.0	53.0	51.7	52.0	52.0	52.0	52.7	55.6	13.5	24.2
456F	1	5	72.7	71.7	72.4	68.7	78.6	73.5	72.4	72.1	70.0	69.7	72.2	2.7	3.8
456F	0.5	2.5	65.4	65.8	67.0	65.20	67.2	64.4	65.0	62.2	68.4	67.8	65.8	1.8	2.8
456F	0.2	1	48.5	44.0	51.0	52.5	46.0	46.5	50.5	57.0	50.5	42.0	48.9	4.4	9.0

Table A4-18: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Corn Bread

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
456K	3	15	118.3	112.0	124.7	112.0	120.0	109.3	119.3	114.3	115.0	115.0	116.0	4.6	3.9
456K	1	5	119.0	122.0	113.0	112.0	121.0	118.0	121.0	119.0	119.0	121.0	118.5	3.4	2.9
456K	0.5	2.5	111.0	110.8	119.8	111.8	113.6	115.0	108.0	105.2	110.6	108.2	111.4	4.1	3.7
456K	0.2	1	61.5	54.5	66.5	58.5	54.5	59.0	58.5	52.0	50.0	50.0	56.5	5.3	9.4

Table A4-19: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Polenta

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
456B	3	15	117.3	110.0	114.3	115.7	114.3	116.3	120.3	119.3	112.3	112.3	115.2	3.2	2.8
456B	1	5	117.0	120.0	118.0	119.0	118.0	118.0	110.0	118.0	64.8	66.6	106.9	21.9	20.5
456B	0.5	2.5	115.4	113.8	116.6	116.4	114.4	112.8	102.6	105.6	109.2	110.0	111.7	4.7	4.2
456B	0.2	1	57.0	58.0	62.5	59.0	61.0	58.5	63.5	65.0	53.5	49.5	58.8	4.7	8.0

Table A4-20: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Masa (dough)

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
418D	3	15	91.0	86.3	94.7	90.0	96.3	93.0	93.3	93.7	83.0	83.3	90.5	4.7	5.2
418D	1	5	109.0	108.0	112.0	109.0	111.0	109.0	108.0	104.0	104.0	99.6	107.4	3.7	3.5
418D	0.5	2.5	108.4	104.8	101.6	96.6	104.6	101.2	108.2	108.2	100.4	101.8	103.6	3.9	3.8
418D	0.2	1	133.5	96.0	94.0	89.5	107.5	99.5	131.0	87.2	89.0	93.5	102.1	17.0	16.6

Table A4-21: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Commercial Taco chips

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
491A	3	15	106.7	129.7	141.0	166.3	147.0	138.7	140.3	147.0	150.3	150.7	141.8	15.6	11.0
491A	1	5	147.0	149.0	151.0	149.0	156.0	157.0	151.0	153.0	157.0	168.0	153.8	6.11	3.97
491A	0.5	2.5	150.8	142.6	155.00	152.4	157.4	158.2	143.2	137.4	152.00	143.2	149.2	7.13	4.78
491A	0.2	1	186.5	172.5	207.0	175.0	179.0	181.0	194.5	186.5	201.5	186.5	187.0	11.16	5.97

Table A4-22: Validation of Sample Extraction and EnviroLogix ELISA with Fortified Non-StarLink Solvent Extracted Germ.

The non-StarLink control sample without any spike gave a value in the assay of 0.432 ng/mL. This matrix effect was reproducible. Therefore, 0.432ng/mL (4.32 ng/g in the sample) was subtracted from all the values obtained in order to generate a the validation table shown below.

Biotech ID #:	Cry-9C ng/ mL spiked	Cry9C ppb spiked	% Cry9C Recovery										Average % Cry9C Recovery	% Cry9C Recovery SD	Cry9C Recovery %CV
		Replicate	1	2	3	4	5	6	7	8	9	10			
457A	6	60	74.63	70.97	71.63	70.63	78.47	77.80	72.97	71.80	74.97	72.13	73.60	2.78	3.78
457A	3	30	67.60	70.27	71.93	72.27	68.27	66.27	69.27	63.60	75.60	63.93	68.90	3.79	5.50
457A	1	10	67.80	52.00	53.70	51.70	51.30	51.60	52.90	58.80	53.10	52.30	54.52	5.14	9.43
457A	0.5	5	43.80	43.20	35.20	27.60	32.80	33.80	52.20	38.80	50.60	48.40	40.64	8.31	20.44

By applying the criteria for LOQ (60% recovery and a CV<25%) to the data in this table, an LOQ of 3ng/mL, corresponding to 30ppb in the sample matrix, could be estimated.

Appendix 5: Instructions for EnviroLogix ELISA kit.

ENVIROLOGIX INC.
Catalog No. AP 008 — Cry9C ELISA Plate Kit

Use of the Kit

The EnviroLogix Cry9C Plate Kit is designed for the quantitative or qualitative laboratory detection of Cry9C endotoxin in corn grain, flour, meal and grits samples. The most prevalent Cry9C varieties in corn are StarLink ™ and StarLink licensees. For ease of reference only, corn modified with Cry9C is referred to throughout this Product Insert as StarLink. Two assay protocols are presented: The High Sensitivity Protocol has a range of 0.01% to 0.125% StarLink corn (by weight) and takes 3 hours to run. The Rapid Protocol has a range of 0.05% to 0.25% StarLink Corn, but takes only 1.75 hours to run.
Please see the enclosed Application Guide for instructions on use of this kit with corn leaf samples and single corn seeds.

How the Kit Works

The EnviroLogix Cry9C Plate Kit is a "sandwich" Enzyme-Linked ImmunoSorbent Assay (ELISA).

In the test, corn product sample extracts are added to test wells coated with antibodies raised against Cry9C toxin. Any residues present in the sample extract bind to the antibodies, and are then detected by addition of enzyme (horseradish peroxidase)-labeled Cry9C antibody.

After a simple wash step, the results of the assay are visualized with a color development step; color development is proportional to Cry9C concentration in the sample extract.

Lighter color = Lower concentration
Darker color = Higher concentration

How the Kit Performs

Limit of Detection
The Limit of Detection (LOD) of the EnviroLogix Cry9C Plate Kit, High Sensitivity protocol, is 0.070 parts per billion (ppb) Cry9C in corn product extract. The LOD was determined by interpolation at 0.079 OD units from a Cry9C standard curve. 0.079 OD units was determined to be 3 standard deviations from the mean of a population of negative corn product samples in the High Sensitivity assay.

The LOD of the EnviroLogix Cry9C Plate Kit, Rapid protocol, is 0.250 ppb Cry9C in corn product extract. The LOD was determined by interpolation at 0.083 OD units from a Cry9C standard curve. 0.083 OD units was determined to be 3 standard deviations from the mean of a population of negative corn product samples in the Rapid assay.

Limit of Quantification
The Limit of Quantification (LOQ) of the EnviroLogix Cry9C Plate Kit was validated at 1.5 ppb in the High Sensitivity assay, and at 6 ppb in the Rapid protocol. The LOQ was determined by fortifying a population of ground corn, cornmeal, corn flour and corn grits samples at the above concentrations of Cry9C protein. In the High Sensitivity protocol, the mean recovery was 101% with a coefficient of variation [CV, (standard deviation/mean) x 100] of 19%. In the Rapid assay, the mean recovery was 110%, with a CV of 15%.

Precision
Cry9C-fortified control solutions were repetitively analyzed both within a single assay (Intra-Assay), and in different assays on different days (Inter-Assay). The data is expressed as % CV for both the recovered concentration (Recov.) and for absorbance (OD).

	Recov. (%CV)	OD (%CV)	Recov. (%CV)	OD (%CV)
	Intra-Assay		**Inter-Assay**	
	High-Sensitivity protocol			
Ctl. 1	8.1%	5.6%	23.8%	n/a
Ctl. 2	3.7%	3.4%	20.1%	n/a
	Rapid protocol			
Ctl. 1	2.4%	2.5%	15.2%	n/a
Ctl. 2	1.7%	1.7%	14.2%	n/a

Fortification and Recovery
For the High Sensitivity protocol, six ground corn, two cornmeal, two corn flour, and two corn grits samples were fortified with Cry9C to a concentration of 6 ppb. The average recovery was 116%, with a CV of 16%.

For the Rapid protocol, six ground corn, two cornmeal, two corn flour, and two corn grits samples were fortified with Cry9C to a concentration of 15 ppb. The average recovery was 113%, with a CV of 9%.

Cross-Reactivity
The EnviroLogix Cry9C Plate Kit does not distinguish between Cry9C endotoxin and certain other compounds, but detects their presence to differing degrees. The following table shows values for the Limit of Detection for a list of compounds. Concentration is in ppb in sample extract.

Compound	High Sensitivity protocol	Rapid protocol
Cry9C	0.070	0.250
Cry1Ab	>50,000	> 100,000
Cry1Ac	>50,000	> 50,000
Cry1C	>50,000	> 100,000
Cry2A	>50,000	> 100,000
Cry1F	66	180

Precautions and Notes

- Store all Plate Kit components at 4° C to 8°C (39°F to 46°F) when not in use.
- Do not expose Plate Kit components to temperatures greater than 37°C (99°F) or less than 2 °C (36°F).
- Allow all reagents to reach ambient temperature (18°C to 27°C or 64°F to 81°F) before use.
- Do not use kit components after the expiration date.
- Do not use reagents or test well strips from one Plate Kit with

reagents or test well strips from a different Plate Kit.

- Do not expose **Substrate** to **sunlight** during pipetting or while incubating in the test wells.
- Do not dilute or adulterate test reagents or use samples not called for in the test procedure.
- Cry9C endotoxin is a protein that can be degraded by heat and sunlight. Samples that cannot be extracted immediately may be stored frozen for up to 1 week prior to analysis.
- It is recommended that results be confirmed by an alternate method.
- Observe any applicable regulations when disposing of samples and kit reagents.

Materials

The EnviroLogix Cry9C Plate Kit contains the following items:

12 strips of 8 antibody-coated wells each, in plate frame
1 vial of Cry9C Negative Control
1 vial of 0.01% StarLink (0.2 ppb Cry9C) Calibrator
1 vial of 0.05% StarLink (1 ppb Cry9C) Calibrator
1 vial of 0.125% StarLink (2.5 ppb Cry9C) Calibrator
1 vial of 0.25% StarLink (5 ppb Cry9C) Calibrator
1 bottle of Cry9C-Enzyme Conjugate
1 bottle 20x Grain Extraction Concentrate
1 packet of Wash Buffer Salts
1 bottle of Substrate
1 bottle of Stop Solution

You will need to provide these items:

- distilled or deionized water for preparing Wash Buffer and diluting 20x Grain Extraction Concentrate
- glass bottles or flasks with 1.2 liter capacity for preparation of 1x Grain Extraction Solution and 1 liter capacity for preparation of Wash Buffer; plus graduated cylinders for measuring the components of these solutions
- test or centrifuge tubes for extraction of grain and dilution of sample extracts
- centrifuge capable of 5000 x g, or low protein-binding hydrophilic syringe filters , 0.45 um (such as Pall Gelman Sciences – Product No. 4184)
- disposable tip, adjustable air-displacement pipettes which will measure 100 and 1000 microliters (µL), and 5 mL
- marking pen (indelible)
- tape or Parafilm®
- timer
- microtiter plate reader or strip reader
- wash bottle, or microtiter plate or strip washer
- multi-channel pipette that will measure 100 µL (optional)
- racked dilution tubes for loading samples into the plate with a multi-channel pipette (optional)
- orbital plate shaker (optional)

Preparation of Solutions

1. **Wash Buffer:**
Add the contents of the packet of **Wash Buffer Salts** (phosphate buffered saline, pH 7.4 - Tween 20) to 1 liter of distilled or deionized water, and stir to dissolve. Store refrigerated when not in use; warm to room temperature prior to assay.

2. **1x Grain Extraction Solution:**
To prepare 1x working Grain Extraction Solution, either:
Mix 5 mL of Grain Extraction Concentrate (20x) plus 95 mL distilled or deionized water for every 100 mL required, or
Add the entire contents of the bottle of Grain Extraction Concentrate (60 mL) supplied in the kit to 1140 mL of distilled or deionized water in a suitable container. Mix thoroughly to dissolve. May be stored at room temperature, but use within 30 days of preparation.

Sample Extraction

Sample Extraction:
Testing of bulk corn grain for Cry9C endotoxin is an indicator of the presence or absence of StarLink GM-modified corn in a given sample. A negative test with this kit is not an indicator of the absence of other genetic modifications.

This protocol calls for a small sample to be analyzed. It is essential that this sample be well mixed and representative of the larger bulk. Note that sampling to detect 0.01% is the equivalent of detecting one kernel of Cry9C corn in a sample of 10,000 kernels.

Note: Thorough mixing of the bulk grain sample and determination of an appropriate sampling plan are critical to the results of this testing, and are the responsibility of the user of this test kit. The USDA/GIPSA has prepared several guidance documents to address the issues involved in obtaining representative grain samples from static lots - such as trucks, barges, and railcars - and for taking samples from grain streams. Sampling plans should be chosen that best meet the needs of both the buyer and seller in terms of acceptable risks. Increasing the number of kernels in the sample and taking multiple samples will increase the likelihood of obtaining representative samples, and maximize the probability of detecting any contamination in the grain lot. For further information on USDA/GIPSA guidelines for obtaining representative samples and assessing detection probabilities for biotech grain, see the following websites:

http://www.usda.gov/gipsa/reference-library/handbooks/grain-insp/grbook1/bk1.pdf
USDA *Grain Inspection Handbook, Book 1, Grain Sampling.* This document provides a comprehensive overview of recommended sampling guidelines for static lots and grain streams. It reviews the various types of equipment and strategies that can be used to obtain a representative grain sample from different types of containers.

http://www.usda.gov/gipsa/biotech/sample2.htm
Guidance document entitled *Sampling for the Detection of Biotech Grains*, which provides important statistical sampling considerations when testing for the presence of biotech grains. It covers the basis for making probability determinations in accepting lots based upon different assumptions with respect to sample size, number of samples, sample preparation, etc.

http://www.usda.gov/gipsa/biotech/sample1.htm
Practical Application of Sampling for the Detection of Biotech Grains. This one-page application guide provides a table that gives sample sizes for selected lot concentrations and probability of rejecting the specified concentrations. It also provides a formula for making the calculation for other combinations.

http://www.usda.gov/gipsa/biotech/samplingplan1.xls
This website provides a simple to use Sample Planner (29k Excel Spreadsheet). The planner allows you to enter different assumptions in terms of sample size, number of samples, acceptable quality level and to determine the probability of accepting lots with given concentration levels. It also plots the probabilities in graph form for easy interpretation. Specific data can be saved for documentation and future analyses.

It is the responsibility of the user to ensure proper sampling and thorough mixing prior to analysis.

Once representative samples have been obtained from the truck or container, they can be reduced in size using a splitter and uniformly ground and mixed.

Note: Sampling and extraction protocols for cornmeal, corn flour, and corn grits are the same as those for ground corn.

1. Weigh 1 gram of ground corn into a 10 mL capacity vial or tube.
2. Add 5 mL of 1x Grain Extraction Solution to each tube. Cap and shake vigorously by hand for 20-30 seconds. Let stand at room temperature to extract; 2-10 minutes of extraction is suitable for screening assays (50-85% of optimal extraction). Optimal extraction requires 3 hours to overnight soaking. Shake again prior to clarification.
3. The extracted samples must be clarified by one of two methods: a) centrifuge the extract at 5000 x g for 5 minutes, or, b) filter the material through a low protein binding hydrophilic syringe filter, 0.45 um (such as Pall Gelman Sciences - Product No. 4184).

Sample Dilution:
Concentrations of Cry9C endotoxin in StarLink corn can range from 8 to 24 micrograms per gram (ppm), and average 10 to 12 ppm. If you are screening bulk grain for the presence or absence of Cry9C and want the maximum sensitivity, you should use undiluted extracts in the High-Sensitivity Protocol described below. If sample extracts produce more color than the highest calibrator and you wish to quantitatively determine the Cry9C endotoxin in that sample you will have to dilute the sample extract in 1x Grain Extraction Solution and run the Rapid Protocol. Multiple dilutions may be required to get the sample extract within the range of calibration. Pure StarLink corn requires at least a 1:1000 additional dilution in order for the extract to be quantitated in the Rapid Protocol. Very strong positive samples may give erroneously low results if they are not diluted sufficiently.

How to Run the Assay

- Read all of these instructions before running the kit.
- Allow all reagents to reach room temperature before beginning (at least 30 minutes with un-boxed strips and reagents at room temperature - do not remove strips from bag with desiccant until they have warmed up).
- Organize all Calibrators and clarified sample extracts, and pipettes so that step 1 can be performed in 15 minutes or less.
- If more than four strips are to be run at one time, the 15 minutes is likely to be exceeded, and the use of a multi-channel pipette is recommended (see "Note" below).
- If four or fewer strips are to be run, use a disposable-tip air-displacement pipette and a clean pipette tip to add each Calibrator and sample extract to the wells. Conjugate, Substrate, and Stop Solution may be added in the same manner; alternatively, use a repeating pipette with a disposable tip on the end of the Combitip for these three reagents.
- If fewer than all twelve strips are used, reseal the unneeded strips and the desiccant in the foil bag provided, and refrigerate.
- Use the well identification markings on the plate frame to guide you when adding the samples and reagents. In a qualitative assay, the Negative Control (NC) and three non-zero calibrators and 88 sample extracts (S) may be run on one plate. (See the Qualitative Assay Example Plate Layout - Figure 1A). For a quantitative assay the Negative Control (NC) and three Calibrators (C1-C3), along with 44 sample extracts (S) may be run in duplicate wells on one plate. (See the Quantitative Assay Example Plate Layout - Figure 1B).

Descriptions of the two assay protocols follow. Choose the one that best fits your needs for detection limits and your time constraints.

The **High Sensitivity Protocol** is for screening of bulk grain, with the ability to detect 0.01% to 0.125% StarLink corn (by weight). This protocol requires 3 hours of total assay incubation time. Only the Negative Control, 0.01% StarLink (0.2 ppb Cry9C), 0.05% StarLink (1 ppb Cry9C), and 0.125% StarLink (2.5 ppb Cry9C) Calibrators should be run in this assay.

The **Rapid Protocol** is slightly less sensitive (able to detect 0.05% to 0.25% StarLink corn) but only requires one hour and 45 minutes of total assay incubation time. Only the Negative Control, 0.05% StarLink (1 ppb Cry9C), 0.125% StarLink (2.5 ppb

Cry9C), and 0.25% StarLink (5 ppb Cry9C) Calibrators should be run in this assay.

NOTE: Estimates of sensitivity and calibrator equivalents in per cent contamination are based upon the assumption that the average StarLink corn expresses 10 ppm of Cry9C endotoxin. Since StarLink corn can express anywhere from 8 to 24 ppm endotoxin, per cent contamination estimates can be expected to vary by 2-fold or more.

HIGH SENSITIVITY PROTOCOL

Procedure

1. For this protocol, use the Negative Control, 0.01% StarLink (0.2 ppb Cry9C), 0.05% StarLink (1 ppb Cry9C), and 0.125% StarLink (2.5 ppb Cry9C) Calibrators. Do not use the 0.25% StarLink (5 ppb Cry9C) Calibrator.

   Add **100 µL** of **Negative Control, 100 µL** of each **Calibrator,** and **100 µL** of each clarified **sample extract** to their respective wells, as shown in the Example Plate Layouts (Figures 1A and 1B). Follow this same order of addition for all reagents.

NOTE: In order to minimize setup time it is recommended that a multi-channel pipette be used in steps 1, 5, 9 and 11 when more than 4 strips are used.

2. Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the benchtop for a full 20-30 seconds. Be careful not to spill the contents!

3. Cover the wells with tape or Parafilm to prevent evaporation and **incubate at ambient temperature** for **30 minutes.** If an orbital plate shaker is available shake plate at 200 rpm.

4. After incubation, carefully remove the covering and vigorously shake the contents of the wells into a sink or other suitable container. Flood the wells completely with Wash Buffer, then shake to empty. Repeat this wash step three times. Alternatively, perform these four washes (300 µL/well) with a microtiter plate or strip washer. Slap the plate on a paper towel to remove as much water as possible.

5. Add **100 µL** of **Cry9C-enzyme Conjugate** to each well.

6. Thoroughly mix the contents of the wells as described in step 2. Be careful not to spill the contents!

7. Cover the wells with new tape or Parafilm to prevent evaporation and **incubate at ambient temperature** for **2 hours**. If an orbital plate shaker is available shake plate at 200 rpm.

8. After incubation, carefully remove the covering and vigorously shake the contents of the wells into a sink or other suitable container. Flood the wells completely with Wash Buffer, then shake to empty. Repeat this wash step three times. Alternatively, perform these four washes (300 µL/well) with a microtiter plate or strip washer. Slap the plate on a paper towel to remove as much water as possible.

9. Add **100 µL** of **Substrate** to each well.

10. Thoroughly mix the contents of the wells, as in step 2. Cover the wells with new tape or Parafilm and **incubate** for **30 minutes at ambient temperature.** Use orbital shaker if available.

Caution: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.

11. Add **100 µL** of **Stop Solution** to each well and mix thoroughly. This will turn the well contents yellow.

NOTE: Read the plate within 30 minutes of the addition of Stop Solution.

RAPID PROTOCOL

Procedure

1. For this protocol, use the Negative Control, 0.05% StarLink (1 ppb Cry9C), 0.125% StarLink (2.5 ppb Cry9C), and 0.25% StarLink (5 ppb Cry9C) Calibrators. Do not use the 0.01% StarLink (0.2 ppb Cry9C) Calibrator.

   Add **100 µL** of **Negative Control, 100 µL** of each **Calibrator,** and **100 µL** of each clarified **sample extract** to their respective wells, as shown in the Example Plate Layouts (Figures 1A and 1B). Follow this same order of addition for all reagents.

NOTE: In order to minimize setup time it is recommended that a multi-channel pipette be used in steps 1, 4, 8 and 10 when more than 4 strips are used.

2. Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the benchtop for a full 20-30 seconds. Be careful not to spill the contents!

3. Cover the wells with tape or Parafilm to prevent evaporation and **incubate at ambient temperature** for **15 minutes.** If an orbital plate shaker is available shake plate at 200 rpm.

4. Add **100 µL** of **Cry9C-enzyme Conjugate** to each well. Do not empty the well contents or wash the strips at this time.

5. Thoroughly mix the contents of the wells as described in step 2. Be careful not to spill the contents!

6. Cover the wells with new tape or Parafilm to prevent evaporation and **incubate at ambient temperature** for **1 hour**. If an orbital plate shaker is available shake plate at 200 rpm.

7. After incubation, carefully remove the covering and vigorously shake the contents of the wells into a sink or other suitable container. Flood the wells completely with Wash Buffer, then shake to empty. Repeat this wash step three times. Alternatively, perform these four washes (300 µL/well) with a microtiter plate or strip washer. Slap the plate on a paper towel to remove as much water as possible.

8. Add **100 µL** of **Substrate** to each well.

9. Thoroughly mix the contents of the wells, as in step 2. Cover the wells with new tape or Parafilm and incubate for 30

minutes at ambient temperature. Use orbital shaker if available.

Caution: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.

10. Add **100 µL** of **Stop Solution** to each well and mix thoroughly. This will turn the well contents yellow.

NOTE: Read the plate within 30 minutes of the addition of Stop Solution.

How to Interpret the Results

Spectrophotometric Measurement

1. Set the wavelength of your microtiter plate reader to 450 nanometers (nm). (If it has dual wavelength capability, use 600, 630 or 650 nm as the reference wavelength.)

2. Set the plate reader to **blank** on the Negative Control wells (this should automatically subtract the mean optical density (OD) of the Negative Control wells from each calibrator and sample OD). If the reader cannot do this, it must be done manually.

3. For a quantitative Cry9C assay, a linear curve fit for the standard curve should be used if the microtiter plate reader you are using has data reduction capabilities. If not, calculate the results manually as described in the "How to Calculate the Quantitative Cry9C Results" section. Be sure to use the appropriate calibrator labels for the Protocol that you ran.

How to Interpret the Semi-Quantitative Results

Compare the OD's of the sample extracts to those of the Calibrators to obtain an estimate of the %StarLink or ppb Cry9C endotoxin in your sample extract. Samples with OD's greater than that of the lowest calibrator are considered positive. Those with OD's lower than that of the lowest calibrator either contain no, or less than 0.01% StarLink (1 ppb Cry9C) in the High Sensitivity Protocol, or less than 0.05% StarLink (5 ppb Cry9C) in the Rapid Protocol.

How to Calculate the Quantitative Cry9C Results

1. After reading the wells, average the OD of each set of calibrators and samples, and subtract the average OD of the Negative Control wells from all.

2. Graph the mean OD of each Calibrator against its % StarLink content (or Cry9C concentration) on a linear scale (see Figures 3a & b). Be sure to label the calibrator levels appropriately for the protocol you ran.

3. Determine the %StarLink content (or Cry9C concentration) of each sample by finding its OD value and the corresponding concentration level on the graph. Multiply the ppb Cry9C result by 5 for the dilution factor incurred during extraction; the %StarLink labels have this dilution taken into account, so do not multiply these by 5. Then multiply ppb Cry9C or %StarLink by any additional dilutions you may have made.

4. Interpolation of sample concentration is only possible if the OD of the sample falls within the range of OD's of the Calibrators.

If the OD of a sample is lower than that of the Low Calibrator (0.2 ppb in the High Sensitivity Protocol, 1 ppb in the Rapid Protocol), the sample must be reported as less than:

High Sensitivity Protocol:
0.01%StarLink corn, or
0.2 ppb x 5 (dilution factor during extraction) = 1 ppb Cry9C.

Rapid Protocol:
0.05% StarLink corn, or,
1 ppb x 5 (dilution factor during extraction) = 5 ppb Cry9C.
If any additional dilutions were performed, multiply by these factors as well.

If the OD of a sample is higher than that of the High Calibrator (2.5 ppb in the High Sensitivity Protocol, 5 ppb in the Rapid Protocol), the sample must be reported as greater than:

High Sensitivity:
0.125% StarLink corn, or,
2.5 ppb x 5 (dilution factor during extraction) = 12.5 ppb Cry9C.

Rapid Protocol:
0.25% StarLink corn, or,
5 ppb x 5 (dilution factor during extraction) = 25 ppb Cry9C.
If any additional dilutions were performed, multiply by these factors as well.

If a concentration must be determined for these high level samples, dilute the sample extract 10 to 1000-fold more than executed in the original assay, in 1x Grain Extraction Solution. Run this dilution in a repeat of the Rapid Protocol. If the result now falls within the range of the OD's of the Calibrators, you must then be sure to use this new dilution factor of sample extract in the calculations described above.

Figure 1A. Example of a typical Qualitative assay setup.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85
B	C1	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86
C	C2	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87
D	C3	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88
E	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	NC
F	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	C1
G	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	C2
H	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	C3

Figure 1B. Example of a typical Quantitative assay setup.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
B	C1	C1	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
C	C2	C2	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
D	C3	C3	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
E	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
F	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
G	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
H	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44

Figure 2a. Illustrative quantitative calculations - High Sensitivity Protocol

Well contents	OD	Average OD ± sd	% CV	Cry9C Concentration (ppb)
Negative Control	0.061 0.061	0.026 ± 0.009	36	NA
0.01%StarLink (0.2 ppb Cry9C) Cal.	0.135* 0.139	0.137 ± 0.003	2.1	NA
0.05% StarLink (1ppb Cry9C) Cal.	0.516* 0.488	0.502 ± 0.020	3.9	NA
0.125% StarLink (2.5 ppb Cry9C) Cal.	1.213* 1.116	1.164 ± 0.069	5.9	NA
Sample**	0.385* 0.375	0.380 ± 0.007	1.9	0.036% StarLink 0.72 ppb Cry9C

* Figures are after subtraction of Negative Control values.
**Concentration from curve = 0.72 ppb Cry9C, multiplied by 1:5 dilution during extraction = 3.6 ppb Cry9C.

Figure 2b.
Illustrative quantitative calculations - Rapid Protocol

Well contents	OD	Average OD ± sd	% CV	Cry9C Concentration (ppb)
Negative Control	0.061 0.061	0.026 ± 0.009	36	NA
0.05%StarLink (1ppb Cry9C) Cal.	0.137* 0.136	0.136 ± 0.001	0.4	NA
0.125% StarLink (2.5 ppb Cry9C) Cal.	0.759* 0.763	0.761 ± 0.003	3.1	NA
0.25% StarLink (5 ppb Cry9C) Cal.	1.460* 1.398	1.429 ± 0.044	3.1	NA
Sample**	1.049 * 1.022	1.035 ± 0.019	1.8	0.17% StarLink 3.46 ppb Cry9C

* Figures are after subtraction of Negative Control values.
**Concentration from curve = 3.46 ppb Cry9C, multiplied by 1:5 dilution during extraction = 17.3 ppb Cry9C.
Actual values may vary; this data is for demonstration purposes only.

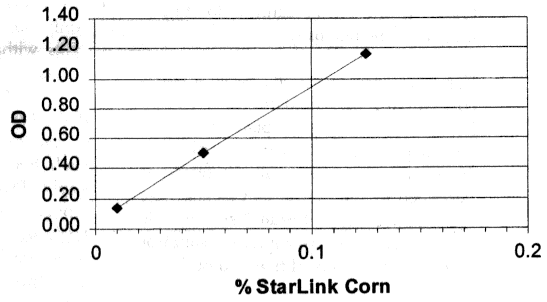
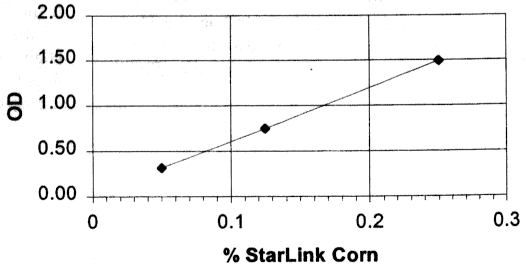


Figure 3a.
Illustrative standard curve - High Sensitivity Protocol

Figure 3b.
Illustrative standard curve - Rapid Protocol



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EnviroLogix Inc. ("EnviroLogix") warrants the products sold hereunder ("the Products") against defects in materials and workmanship when used in accordance with the applicable instructions for a period of one year from the date of shipment of the products or if shorter, for a period not to extend beyond a product's printed expiration date. If the Products do not conform to this Limited Warranty and the customer notifies EnviroLogix in writing of such defects during the warranty period, including an offer by the customer to return the Products to EnviroLogix for evaluation, EnviroLogix will repair or replace, at its option, any product or part thereof that proves defective in materials or workmanship within the warranty period.

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Rev. – 11/27/00

For Technical Assistance please contact:

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Appendix 6: Certificate of analysis

Biotechnology Support
Aventis CropScience
2 TW Alexander Drive
RTP, NC 27709
USA

No. BTS-0007/01
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CERTIFICATE OF ANALYSIS NO. BTS-0007/01

General

This Certificate of Analysis fulfills the requirement for the characterization of sample material used in a study. It documents the identity and purity/content of the sample.

Designation of the Certified Material:

Material:	StarLink (CBH351) and Non StarLink Corn Grain, Masa, Tortilla, and Tortilla Chips
Code No.:	CM00B010
Batch No.:	BTID 414I, 418C, 414C, 418D, 414A, 418N, 414B, 418M
Sample No.:	NA

Origin of the Certified Material

Cereal Quality Lab
Texas A&M University
2474 TAMUS
College Station, TX 77843-2474

Methods

The ☒ Identity
☒ Purity
☐ Content

of the material was established by use of the following method(s):

☒ Discriminating PCR
☐ Southern Blotting
☐ ELISA (Method)
☐ Bradford Assay (Method)

Date of Analysis

December 19, 2000

Biotechnology Support
Aventis CropScience
2 TW Alexander Drive
RTP, NC 27709
USA

Results

DNA was prepared from samples received from Texas A&M University. Each corn grain sample consisted of approximately 400 kernels. Each corn product sample was prepared from 40 to 160 g of the corresponding material. Each matrix was ground according to SOP 6001.01, and duplicate DNA extractions were performed using the Qiagen plant extraction kit. Discriminating PCR for event CBH351 was performed on each extraction, in duplicate. Results for PCR analyses are shown in Table 1.

Results from the discriminating PCR analysis of the transgenic corn grain and products showed the expected pattern for StarLink (CBH 351 event), confirming the identity of the initial corn grain sample. The non-StarLink (NT) grain and corn products were found to have only the endogenous control PCR product, confirming that they are not event CBH 351, and that this material will serve as a proper control for this study.

TABLE 1
CBH351 dPCR for corn products

	BT ID #	Number positive/total
Transgenic grain	414I	2/2
NT grain	418C	0/2
Transgenic Masa	414C	2/2
NT Masa	418D	0/2
Transgenic Tortillas	414A	2/2
NT Tortillas	418N	0/2
Transgenic Tortilla Chips	414B	2/2
NT Tortilla Chips	418M	0/2

Biotechnology Support
Aventis CropScience
2 TW Alexander Drive
RTP, NC 27709
USA

Testing Facility

Biotechnology Support
Aventis CropScience
2 TW Alexander Dr.
RTP, NC 27709
USA

Raw data are archived at Aventis CropScience, RTP, NC.

	Name (Typed)	Signature	Date (M/D/Y)
Responsible Scientist:	Ali Scott		3/29/01
Authorized by:	Raymond D. Shillito		3/29/01

Appendix 7: Dry Milling Report

SPONSOR:

Aventis CropScience
Aventis Crop Science Research Center
Research Triangle Park, North Carolina

STUDY DIRECTOR:

William J. Kowite, Ph.D.

REPORT:

Corn: Dry Milling

STUDY TITLE:

Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods

AUTHOR:

Dick Dusek

Dick Dusek
Signature

4/2/01
Date

PROCESSING FACILITY:

GLP Program
Texas A & M University
Food Protein Research and Development Center
Highway 47, Building 8525
Bryan, TX 77801

STUDY IDENTIFICATION:

Study Number: CM00B010

GLP COMPLIANCE STATEMENT

PROJECT TITLE: Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods

This processing study was conducted and reported in accordance with the Environmental Protection Agency's Good Laboratory Practice Standards, 40 CFR 160, Federal Register, effective date October 16, 1989.

Dick Dusek
Processing Principal Investigator

4/2/01
Date

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: **Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods**

In compliance with the Good Laboratory Practice regulations an inspector with the Quality Assurance Unit has inspected at least one phase of this study. Inspection findings were reported to GLP Program management, the study director and the study director's management. The Quality Assurance Unit has reviewed the processing report and certifies that it accurately describes the methods and standard operating procedures used, and the reported results accurately reflect the raw data generated during this processing phase.

Signed: _____ Date: 02 Apr 2001

Doyle L. Borchgardt
Quality Assurance Coordinator
Food Protein Research and Development Center

INSPECTION		DATES REPORTED TO:	
TYPE	DATE	GLP PROGRAM MANAGEMENT	STUDY DIRECTOR & STUDY DIRECTOR'S MANAGEMENT
1) Process Phase SOP 8.6 R11, Section 4: "Solvent Extraction of Germ Oil"	02 & 03 Jan 2001	09 Jan 2001	24 Jan 2001
2) Process Report	27 thru 30 Mar 2001	30 Mar 2001	02 Apr 2001

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PROJECT TITLE: **Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods**

SPONSOR: Aventis CropScience
Aventis Crop Science Research Center
Research Triangle Park, North Carolina

STUDY DIRECTOR: William J. Kowite, Ph.D.

PRINCIPAL INVESTIGATOR: Dick Dusek

PROCESSING, DATA RECORDING & SHIPPING TECHNICIANS: Dick Dusek, Pat Simecek, Derrold Foster, Adam Hyman Jr., Randy Gaas, and Crystal Perkkio.

SAMPLE RECEIPT DATE: Control: 10-30-00 and 11-13-00
Starlink™: 10-30-00 and 11-9-00

PROCESSING START DATE: 11-13-00

PROCESSING TERMINATION DATE: 1-3-01

FRACTION SHIPMENT DATE:
11-16-00 (Cereal Quality Laboratory Samples)
11-21-00 (Aventis CropScience-Kowite)
12-1-00 (Diehl, Incorporated)
12-1-00 (F.R.I. Enterprises)
12-18-00 (Aventis CropScience-Artis)
12-18-00 (General Mills)
12-19-00 (Aventis CropScience-Shillito)
12-19-00 (Aventis CropScience-Kowite-Corn Flakes)
1-2-01 (The National Food Laboratory, Inc.)
1-9-01 (Wildlife International, Ltd.)
1-24-01 (Aventis CropScience-Artis)

INTRODUCTION:

Corn samples were received from Qualls Agricultural Laboratory in Ephrata, Washington (Control) and Land O'Lakes Research Farm in Webster City, Iowa (Starlink™) and were processed into commercially representative fractions. These fractions were sent to Aventis CropScience in Research Triangle Park, North Carolina; Wildlife International, Ltd. in Easton, Maryland; The National Food Laboratory, Incorporated in Dublin, California; General Mills in Minneapolis, Minnesota; F.R.I. Enterprises in New Berlin, Wisconsin; and Diehl, Incorporated in Defiance, Ohio.

TEST SUBSTANCE: [From protocol and/or MSDS]

None

OBJECTIVE:

The objective of this processing facility was to generate commercially representative processed fractions from corn samples grown in the field.

METHODS & MATERIALS:

Sample Receipt:

All corn grain samples (RAC/seed) were received at ambient temperature at the Food Protein Research and Development Center's GLP Program in Bryan, Texas. Control samples were received 10-30-00, and 11-13-00. Starlink™ samples were received 10-30-00 and 11-9-00. Control samples were shipped 10-26-00, 11-8-00, and 12-18-00 respectively by Qualls Agricultural Laboratory in Ephrata, Washington via Federal Express. Starlink™ were shipped 10-26-00 and 11-7-00 respectively by Land O'Lakes Research Farm in Webster City, Iowa (Starlink™) via Federal Express. Control samples shipped 11-8-00 were transferred from Federal Express to AFTCO Enterprises, Incorporated in Houston, Texas on 11-13-00 and delivered to the GLP Program. The samples were identified and processed in the following order: CM00B010-04 (Control) and CM00B010-03 (Starlink™). Control samples received on 10-30-00 were labeled as CM00B010-04-TX. All Starlink™ samples received were labeled as CM00B010-03-TX. .

Storage Conditions:

Corn samples received 10-30-00 (control and Starlink™) were placed in temporary freezer "D". At the request of the Study Director, the samples were removed on 11-7-00 and stored ambient. All samples and collected fractions were stored ambient in room 123 of building 8525.

Sample/Fraction Handling:

Samples were handled in a manner that minimizes the possibility of contamination. It is this facility's policy to use only containers and utensils washed with detergent and rinsed with water.

Processing Methods:

Whole corn is dried (if necessary) in a Proctor Schwartz oven at 130-160°F to a moisture content of 10-15%. The light impurities are separated using a Kice aspirator. After aspiration, the sample is screened in a Vac-Away two screen cleaner to separate large and small foreign particles (screenings) from the corn.

The whole corn grain is moisture conditioned to 20-22% and allowed to "temper" for 2-2.5 hours. After tempering, the corn grain is impact milled in a Ripple mill. After milling, the cornstock is dried at 130-160°F for 30 minutes. Cornstock is allowed to cool to approximately 90°F after removal from the oven. The cornstock is passed over a 1/8" shaker screen. Material above the screen is further processed into large grits,

germ, and hull (bran). Material through the screen is separated into medium and small grits, coarse meal, meal, and flour.

The material above the 1/8" screen is passed through a Kice aspirator to separate the hull material and hull material with attached germ from the large grits and germ. The hull material and hull material with attached germ is aspirated at a lower setting to separate the hull material from the hull material with attached germ. Hull material with attached germ is passed through the Ripple mill and aspirated to separate the hull from the germ. The hull material is combined. Large grits and germ from the first aspiration are separated on an Oliver gravity separator. The germs are combined and dried at 130-160°F to 8-12% moisture.

The material passing through the 1/8" shaker screen is separated using a Great Western sample sifter. The sifter is fitted with the following screen sizes: 0.0800", 0.0540", 0.0204", and 0.0098". Material on top of the 0.0800" screen is medium grits; material on top of the 0.0540" screen is small grits; material on top of the 0.0204" screen is coarse meal; material on top of the 0.0098" screen is meal; and material through the 0.0098" screen is flour.

The germ is heated to 160-175°F and held in this range for 10 minutes. Heated germ is flaked in a Ferrell Ross flaking roll with a gap setting of 0.008 to 0.012" and promptly taken to solvent extraction.

The flaked germ is placed in stainless steel batch extractors and submerged in 120-140°F solvent (hexane). After 30 minutes, the hexane is drained and fresh hexane added to repeat the cycle two more times. The final two washings are for 15 minutes each. After the final draining, warm air is forced through the extracted presscake to remove residual hexane.

Miscella (crude oil and hexane) is passed through a Precision Scientific Recovery unit to separate the crude oil and hexane. Crude oil is heated to 163-194°F for hexane removal. The crude oil recovered from solvent extraction is sampled, and refined according AOCS method Ca9a52.

After refining, the refined oil and soapstock are separated. The refined oil can be further bleached, and deodorized.

This processing procedure is outlined in form 300.6 (Material Balance of Dry Corn) and is described in detail in SOP 8.6 Revision 11, "Small-Scale Dry Milling of Corn." Additional processing included production of corn flakes. Following is a description of the process.

PRODUCTION OF CORN FLAKES

Grits

Large grits produced from dry milling to be used for corn flakes should be # 4 or 5 in size, however a size of # 7 (or close to 1/8") is permissible for batch processing. The larger grit will better retain its identity during the process. Actual grit size was greater than 11/64" or approximately a #4.

Starting grit moisture should be approximately 11.7% (range of 10.0 to 12.5 %). If corn is presently being milled for grits, the starting moisture level is not critical. The lower moisture level is for storage of grits. Actual starting moisture was 14.7% (control) and 15.3% (Starlink™).

Formulation

A variety of formulations are used to produce commercial corn flakes. One typical, basic formulation is as follows: grits blended with 6% granulated sugar, 2% malt syrup and 2% salt by weight. The flavoring ingredients are mixed with a sufficient quantity of water to provide uniform dispersion or proportional mix with the grits.

Note: The sponsor must indicate what ingredients are to be used with the grits.

Used only grits and water per sponsor instructions.

Cooking

A weighed amount of grits were soaked in reverse osmosis water for 18 minutes for a final moisture of 22.4% for both samples. The product was cooked with steam and under a pressure of 15-18 psi in a pressure cooker for 16 minutes with a maximum temperature of 256.5°F (control) and 255°F (Starlink™).

After pressure cooking any clumps of grits were broken up and the moisture content of the grits was checked (should be between 28 to 32 %). The final moisture of the cooked grits was 31.4% (control) and 31.9% (Starlink™). Cooking changed the grit's appearance from hard, chalky white to a soft, translucent and light golden brown.

Drying

Grits were dried at maximum temperatures of 154 °F (control) and 156 °F (Starlink™) until the moisture content was 22.5 % (control) and 24.2% (Starlink™). After removing the grits from

the drier, grits were promptly cooled for 5 minutes at a temperature of 39.5°F (control) and 40.2°F (Starlink™). The grits were sealed in a plastic bag (at ambient temperature) and allowed to equilibrate for approximately 19 hours (control) and 21.5 hours (Starlink™).

Flaking

Prior to flaking, a sufficient amount of steam (less than 5 psi) was applied to the grits for a period less than one minute to make their surface area sticky.

With a preset roll gap at 0.004 of an inch, the grits were slowly fed through flaking rolls. A system to scrape the flakes off the rolls was in place.

Toasting

Flakes (single layered on a stainless steel screen) were placed in a preheated oven and toasted 2.5 minutes at a temperature between 525 and 575°F. The final moisture content of the toasted flakes was 5.7% (control) and 7.7% (Starlink™).

References

The above batch method was developed from the two references below.

Fast, R. B.. and Caldwell, E. F., eds. 1991. Breakfast Cereals and How They Are Made. Am. Assoc. Cereal Chem., St. Paul, MN.

Matz, S.A., 2nd ed. 1991. The Chemistry and Technology of Cereals as Food and Feed. Van Nostrand Reinhold/AVI, New York, NY.

Differences in this batch method as compared to commercial practice and indicated in the text are as follows:

* Ingredients, such as granulated sugar, malt syrup, salt, vitamins and minerals, were not added.
* Temper time of cooked grits is shorter due to modern equipment which accelerates moisture equilibration.
* Commercial flaking rolls are set at 180 to 200 rpm with a roll differential of 6 to 8%. Our rolls were set at 400 rpm with no differential.
* Commercial toasting is performed in rotary ovens. Our method of toasting was stationary.
* The final moisture of commercial, toasted flakes is less than 3%. Our flake moisture was a little higher.

Comparison to Industrial Practice:

Dry milling by the GLP Program very closely simulates commercial dry milling practices. Slight variations in industrial milling practices are designed to suit the buyer's needs.

The majority of commercial plants will remove the oil from the germ by expelling (hardpressing). A small percentage will utilize direct solvent extraction to remove the crude oil. Due to equipment available to the GLP Program, hardpressing is not possible.

In comparison, the program's goal is to produce the same component parts for each sample within a study to be used in residue determination. Because of compliance monitoring requirements and sample size, the samples were processed by batch rather than continuous, as in commercial operation.

Processing Results:

Whole corn was dry milled into hull, germ, large grits, medium grits, small grits, coarse meal, meal, flour, and flaked germ after solvent extraction (meal). An unprocessed sample was taken before processing. All fractions collected during this study are listed in the original raw data.

Other Circumstances Pertaining to Study:

The following facility SOP deviations were reported to the Study Director via facsimile:

1. Maximum oven temperature during dry milling of control sample on 12-18-00 was not recorded. Time samples removed from freezer/room were not recorded. Due to multiple batches, fraction collection times were not recorded.

2. After initial pass through Ripple mill, corn stock was dried for 120-124 minutes (except control batch milled 11-20-00 and 0.3% "spiked" sample).

3. A Dynascreen classifier was used to separated medium and small grits, coarse and regular meal, and flour instead of the Great Western Sifter.

4. Pre-process verification and cleaning are not recorded for the Ferrell Ross flaking roll used during production of corn flakes.

5. The Great Western Sifter was used on 11-20-00. Pre-process verification and cleaning were recorded on 11-

21-00.

Samples of control and Starlink™ corn were delivered on 11-9-00 to Dr. Lloyd Rooney at the Cereal Quality Laboratory at Texas A&M University.

Dr. William Kowite (Study Director) visited the GLP Program and Cereal Quality Laboratory on 11-10-00.

After separation of fractions from standard dry milling process, the large grits, medium grits, small grits, and coarse meal were ground with a Fitzpatrick hammermill and separated on the Dynascreen classifier to produce meal and flour.

At the request of Dr. Raymond Shillito (Sponsor), the "spiked" sample was increased from 0.1% to 0.3%. On 12-14-00, 306.5 grams of Starlink™ was added to 225.0 lbs. of control corn and mixed for 2 hours and 7 minutes. This sample was identified as CM00B010-05. After mixing the "spiked" sample was dry milled.

Fraction Shipment:

Ambient processed corn fractions were shipped priority overnight by Federal Express. Fractions shipped 1-24-01 were shipped second day by Federal Express. A Chain of Custody accompanied each fraction shipment. Refer to page 5 of this report for shipment dates and locations.

CONCLUSIONS:

Control and Starlink™ corn grain samples were processed into commercially representative fractions.

DATA ARCHIVAL:

Record Transfer and Retention:

This processing report as listed in the table of contents has been sent via overnight letter or package to William J. Kowite, Ph.D. at Aventis CropScience in Research Triangle Park, North Carolina for archiving.

The Food Protein Research and Development Center will archive the following study specific data:

-copy of the sponsor processing protocol
-exact copy of the processing report (main body)
-exact copy of the compliance statement
-exact copy of the sample material balance
-exact copy of the original raw processing data (includes communication logs, calculations, and deviation forms, when applicable)
-exact copy of personnel records (names and initials of personnel with processing study duties)
-exact copy of receiving record(s)
-exact copy of shipping record(s)
-exact copy of shipping bill of lading(s)

The Food Protein Research and Development Center will archive the following non-study specific data indefinitely:

-original freezer temperature records
-original equipment logs (includes scales, temperature recording devices, and processing equipment records)
-CVs of personnel and training records

REVISION# 04 FORM# 300.6

MATERIAL BALANCE of DRY CORN

Sample # **1 (Control)** Code # **CM00B010-04**

WHOLE CORN 1590.4 lbs

Drying — 1512.0 lbs (after drying)

Aspiration — 31.2 lbs **LIGHT IMPURITIES**

Screening — 8.5 lbs **SMALL SCREENINGS**
0.9 lbs **LARGE SCREENINGS**

800.0 lbs Dry Milled

Steeping — 80.4 lbs water added

Degermination, Drying, Screening, Aspiration, and Separation

385.7 lbs **LARGE GRITS***	63.1 lbs **COARSE MEAL***
84.0 lbs **MEDIUM GRITS***	28.1 lbs **MEAL**
66.5 lbs **SMALL GRITS***	46.4 lbs **FLOUR**
58.4 lbs **HULL MATERIAL**	

71.2 lbs **GERM** (Dried to 65.3 lbs)

Conditioning, & Flaking — 65.3 lbs Germ Conditioned

n/a g **CRUDE OIL** 48.1 lbs **SOLVENT EXTRACTED**

- Ground with Fitzpatrick hammermill and sifted on Dynascreen separator to produce meal and flour. Final results (including initial meal and flour recovered before grinding):

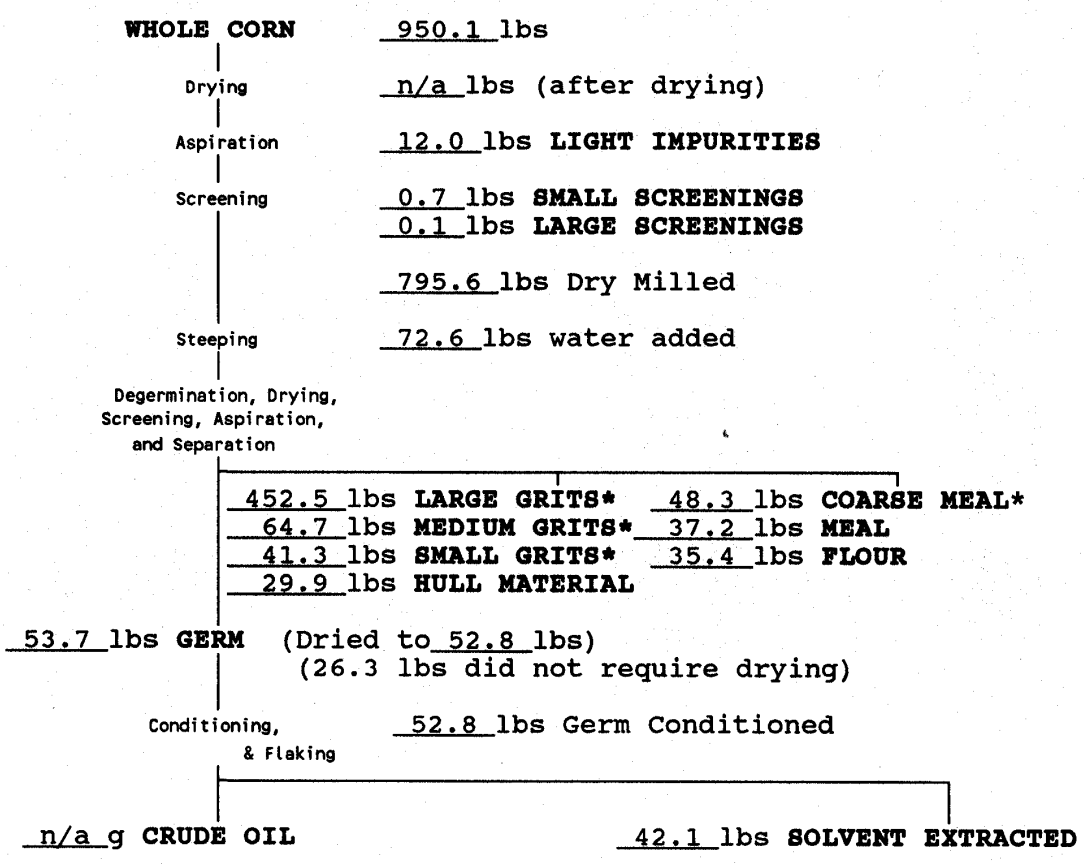
  366.9 lbs Meal
  187.9 lbs Flour
  67.3 lbs Material not grinding into meal or flour

Note: On 12-18-00, 23.1 lbs of water was added to 225.0 lbs of corn and conditioned for 2 hours and 3 minutes. The corn was then dry milled and produced 18.8 lbs of large grits for corn flake production. 3.1 lbs of large grits were used to produce 1.1 lbs of Toasted Corn Flakes.

REVISION# 04 FORM# 300.6

MATERIAL BALANCE of DRY CORN

Sample # **2 (Starlink™, CBH351)** Code # **CM00B010-03**



- Ground with Fitzpatrick hammermill and sifted on Dynascreen separator to produce meal and flour. Final results (including initial meal and flour recovered before grinding):

  383.8 lbs Meal
  219.2 lbs Flour
  10.5 lbs Material not grinding into meal or flour

Note: On 12-18-00, 12.0 lbs of water was added to 126.6 lbs of corn and conditioned for 2 hours and 3 minutes. The corn was then dry milled and produced large grits for corn flake production. 3.0 lbs of large grits were used to produce 1.4 lbs of Toasted Corn Flakes.

Note: Large grits produced on 11-30-00 and included with this material balance are estimated.

REVISION# 04 FORM# 300.6

MATERIAL BALANCE of DRY CORN

Sample # **3 (0.3% "Spiked")** Code # **CM00B010-05**

WHOLE CORN n/a lbs

Drying — n/a lbs (after drying)

Aspiration — n/a lbs **LIGHT IMPURITIES**

Screening — n/a lbs **SMALL SCREENINGS**
n/a lbs **LARGE SCREENINGS**

225.7 lbs Dry Milled

Steeping — 22.2 lbs water added

Degermination, Drying, Screening, Aspiration, and Separation

92.6 lbs **LARGE GRITS***	17.4 lbs **COARSE MEAL***
23.0 lbs **MEDIUM GRITS***	10.4 lbs **MEAL**
21.3 lbs **SMALL GRITS***	15.2 lbs **FLOUR**
14.5 lbs **HULL MATERIAL**	

32.9 lbs **GERM** (Dried to 30.8 lbs)

Conditioning, & Flaking — 30.8 lbs Germ Conditioned

n/a g **CRUDE OIL** 24.9 lbs **SOLVENT EXTRACTED**

- Ground with Fitzpatrick hammermill and sifted on Dynascreen separator to produce meal and flour. Final results (including initial meal and flour recovered before grinding):

  69.7 lbs Meal
  72.4 lbs Flour
  2.3 lbs Material not grinding into meal or flour

APPENDIX

Raw Data in support of this study are available under separate cover.

Appendix 8: Wet Milling Report

SPONSOR:

Aventis CropScience
Aventis Crop Science Research Center
Research Triangle Park, North Carolina

STUDY DIRECTOR:

William J. Kowite, Ph.D.

REPORT:

Corn: Wet Milling

STUDY TITLE:

Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods

AUTHOR:

Dick Dusek

Dick Dusek
Signature

4/2/01
Date

PROCESSING FACILITY:

GLP Program
Texas A & M University
Food Protein Research and Development Center
Highway 47, Building 8525
Bryan, TX 77801

STUDY IDENTIFICATION:

Study Number: CM00B010

GLP COMPLIANCE STATEMENT

PROJECT TITLE: Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods

This processing study was conducted and reported in accordance with the Environmental Protection Agency's Good Laboratory Practice Standards, 40 CFR 160, Federal Register, effective date October 16, 1989.

Dick Dusek
Dick Dusek
Processing Principal Investigator

4/2/01
Date

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: **Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods**

In compliance with the Good Laboratory Practice regulations an inspector with the Quality Assurance Unit has inspected at least one phase of this study. Inspection findings were reported to GLP Program management, the study director and the study director's management. The Quality Assurance Unit has reviewed the processing report and certifies that it accurately describes the methods and standard operating procedures used, and the reported results accurately reflect the raw data generated during this processing phase.

Signed: _____ Date: 02 Apr 2001

Doyle L. Borchgardt
Quality Assurance Coordinator
Food Protein Research and Development Center

INSPECTION		DATES REPORTED TO:	
TYPE	DATE	GLP PROGRAM MANAGEMENT	STUDY DIRECTOR & STUDY DIRECTOR'S MANAGEMENT
1) Process Phase SOP 8.13 R. 07: "Laboratory Deodorization of Vegetable Oil"	08 Jan 01	09 Jan 2001	24 Jan 2001
2) Process Report	27 thru 30 Mar 2001	30 Mar 2001	02 Apr 2001

TABLE OF CONTENTS

PROJECT TITLE: **Detection of Cry9C Protein in Wet Milled, Dry Milled and Masa based Processed Fractions and Foods**

SPONSOR: Aventis CropScience
Aventis Crop Science Research Center
Research Triangle Park, North Carolina

STUDY DIRECTOR: William J. Kowite, Ph.D.

PRINCIPAL INVESTIGATOR: Dick Dusek

PROCESSING, DATA RECORDING & SHIPPING TECHNICIANS: Dick Dusek, Pat Simecek, Adam Hyman Jr., Carolyn Hines, Randy Gaas, and Crystal Perkkio.

SAMPLE RECEIPT DATE: Control: 12-18-00 and 12-20-00
Starlink™: 12-18-00 and 12-19-00 (1 box)

PROCESSING START DATE: 12-23-00

PROCESSING TERMINATION DATE: 1-8-01

FRACTION SHIPMENT DATE: 1-2-01 and 1-9-01

INTRODUCTION:

Corn samples were received from Qualls Agricultural Laboratory in Ephrata, Washington (Control) and Land O'Lakes Research Farm in Webster City, Iowa (Starlink™) and were processed into commercially representative fractions. These fractions were sent to Aventis CropScience in Research Triangle Park, North Carolina.

TEST SUBSTANCE: [From protocol and/or MSDS]

None

OBJECTIVE:

The objective of this processing facility was to generate commercially representative processed fractions from corn samples grown in the field.

METHODS & MATERIALS:

Sample Receipt:

All corn grain samples (RAC/seed) were received at ambient temperature at the Food Protein Research and Development Center's GLP Program in Bryan, Texas. Control samples were received 12-18-00 and 12-20-00. Starlink™ samples were received 12-18-00 and 12-19-00 (1 box). Control samples were shipped 12-15-00 and 12-18-00 respectively by Qualls Agricultural Laboratory in Ephrata, Washington via Federal Express. Starlink™ were shipped 12-15-00 by Land O'Lakes Research Farm in Webster City, Iowa (Starlink™) via United Parcel Service (UPS). The samples were identified and processed in the following order: CM00B010-04 (Control) and CM00B010-03 (Starlink™). Starlink™ samples received were labeled as CM00B010-03-TX2.

Storage Conditions:

All samples and collected fractions were stored ambient in room 123 of building 8525.

Sample/Fraction Handling:

Samples were handled in a manner that minimizes the possibility of contamination. It is this facility's policy to use only containers and utensils washed with detergent and rinsed with water.

Processing Methods:

The whole corn samples are dried (if necessary) in a Proctor Schwartz oven between 130-160°F. The final moisture content after drying is between 10-15% The light impurities are separated using a Kice aspirator. After aspiration, the sample is screened in a Vac-Away two screen cleaner. Large and small foreign particles (screenings) are separated from the corn.

The cleaned corn is steeped in 120-130°F water containing 0.1-0.2% sulfur dioxide (sulfurous acid) for 22-48 hours. At the end of the steeping period, the whole corn is passed through a Bauer mill with devil toothed plates and a majority of the germ and hull are removed using a hydroclone. Germ and hull are dried at 165-195°F to obtain a final moisture between 5-10%. After drying, the germ and hull are separated using aspiration.

The cornstock (without germ and hull) is ground in a Rietz mill with a 0.023" screen. The material going through the 0.023" screen is passed through a Dynascreen equipped with a 43-micron screen. Material on top of the screen is a product of batch processing and is discarded. In commercial industry, only bran

(hull material) remains on top of the screen. The process water (with starch and gluten) passing through the 43-micron screen is separated into component parts using batch centrifugation.

The germ is moisture conditioned to 12%, heated to 190-220°F, flaked in a Ferrell-Ross flaking roll with a gap setting of 0.008 to 0.012", and pressed in a Rosedown expeller to liberate part of the crude oil. Resulting fractions are expelled crude oil and presscake with residual crude oil.

The presscake is placed in stainless steel batch extractors and submerged in 120-140°F solvent (hexane). After 30 minutes, the hexane is drained and fresh hexane added to repeat the cycle two more times. The final two washings are for 15-30 minutes each. After the final draining, warm air is forced through the extracted presscake to remove residual hexane.

The miscella (crude oil and hexane) is passed through a Precision Scientific Recovery unit to separate the crude oil and hexane. Crude oil is heated to 163-194°F for hexane removal.

The crude oil recovered from expelling and solvent extraction is combined, sampled, and refined according to AOCS method Ca9a52. After refining, the refined oil and soapstock are separated. The refined oil can be further bleached, and deodorized.

This processing procedure is outlined in form 300.5 (Material Balance of Wet Corn) and is described in detail in SOP 8.5 Revision 12, "Small-Scale Wet Milling of Corn"; SOP 8.11 Revision 07, "Laboratory Bleaching of Oil"; and SOP 8.13 Revision 07, "Laboratory Deodorization of Oil."

Comparison to Industrial Practice:

The corn was wet milled in a way that simulates industrial practice as closely as possible. Because of compliance monitoring requirements and sample size, the samples were processed by batch rather than continuous, as in commercial operation.

Due to equipment limitations and batch processing the material balance values for wet milling products will be estimated using percentages from the CRC Handbook of Processing and Utilization in Agriculture. Fraction yields obtained by the industry are not made public. Yields from commercial wet milling plants will vary between plants depending on quality of the corn and differences in milling practice and fiber and germ washing operations. The following table is for approximate yields.

Solubles from steeping	-	7.5%
Starch	-	67.5%

Gluten	-	5.8%
Germ	-	7.5%
Hull	-	11.5%

Processing Results:

Wet milled corn samples were processed into hull, germ, gluten, starch, presscake from the expeller, crude oil from the expeller, presscake after solvent extraction, crude oil after solvent extraction, refined oil, soapstock, bleached oil, and deodorized oil. An unprocessed sample (RAC) was taken before processing. All fractions collected during this study are listed in the original raw data.

Other Circumstances Pertaining to Study:

The following protocol amendment deviation was reported to the Study Director via facsimile:

1. Equipment was to be thoroughly cleaned and inspected prior to processing samples containing CBH351. Cleaning and inspection are not recorded in the data or logs.

The following facility SOP deviations were reported to the Study Director via facsimile:

1. Vacuum gauge VG95-4 used during bleaching and deodorization was not standardized prior to use.

2. Starlink® germ was dried to 4.9% moisture.

3. Pre-process verification and cleaning are not recorded for the following equipment: a.) Precision Scientific Recovery Units 1 and 2 during steepwater concentration; b.) Proctor-Schwartz oven 1 used for drying starch; c.) Bauer Wet Mill; and d.) Precision Recovery Unit 1 used during crude oil recovery.

4. The following deviations from data collection occurred: a.) Time samples removed and placed in freezer were not recorded; b.) Time fractions collected were not recorded in most cases; c.) Sulfurous Acid used to steep Starlink® corn was not recorded in dispensing log; and d.) Sodium Hydroxide (16° Baume) used to refine crude oil was not recorded in dispensing log.

Dr. William Kowite (Study Director) visited the GLP Program and Cereal Quality Laboratory on 11-10-00.

After separation, starch and gluten were dried in a Proctor-Schwartz oven at 165-195°F. Drying was performed since fractions were stored at ambient conditions.

Original temperature charts for storage of samples and collected fractions are included with the dry mill portion of this study. There are no charts for storage of steepwater samples in the cooler.

Control samples received 12-20-00 were not dried, cleaned, or used in processing.

Fraction Shipment:

Ambient processed corn fractions were shipped priority overnight by Federal Express to Aventis CropScience in Research Triangle Park, North Carolina on 1-2-01 and 1-9-01. A Chain of Custody accompanied fraction shipment.

CONCLUSIONS:

Control and Starlink™ corn grain samples were processed into commercially representative fractions.

DATA ARCHIVAL:

Record Transfer and Retention:

This processing report as listed in the table of contents has been sent via overnight letter or package to William J. Kowite, Ph.D. at Aventis CropScience in Research Triangle Park, North Carolina for archiving.

The Food Protein Research and Development Center will archive the following study specific data:

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-exact copy of shipping record(s)
-exact copy of shipping bill of lading(s)

The Food Protein Research and Development Center will archive the following non-study specific data indefinitely:

-original equipment logs (includes scales, temperature recording devices, and processing equipment records)
-CVs of personnel and training records

REVISION# 05 FORM# 300.5

MATERIAL BALANCE of WET CORN

Sample # **1 (Control)** Code # **CM00B010-04**

WHOLE CORN 390.1 lbs

Drying n/a lbs after drying

Aspiration 18.9 lbs **LIGHT IMPURITIES**

Screening 6.9 lbs **SMALL SCREENINGS**
0.5 lbs **LARGE SCREENINGS**

Steeping 175.0 lbs Corn Steeped
35 gal water added

Draining 271.9 lbs Steeped Corn
22 gal **STEEPWATER**

13.1 lb Solubles from steeping*

Degermination, Separation, Screening, and Water Washing

13.1 lbs **GERM*** 20.1 lbs **HULL***
10.2 lbs **GLUTEN***
118.1 lbs **STARCH***

Flaking, Conditioning, & Expelling 10.5 lbs germ pressed
335.9 g water added

960.3 g **CRUDE OIL** 7.9 lbs **PRESSCAKE**

Solvent Extraction

1054.0 g **CRUDE OIL** 5.4 lbs **SOLVENT EXTRACTED PRESSCAKE**

1407.0 g Refined 57.6 g NaOH added

1200.6 g **REFINED OIL** **SOAPSTOCK** 96.2 g

1200.6 g Bleached

1140.3 g **BLEACHED OIL** **

1113.2 g Deodorized

1090.9 g **DEODORIZED OIL** ** 325.6 g **DEODORIZER DISTILLATES** **

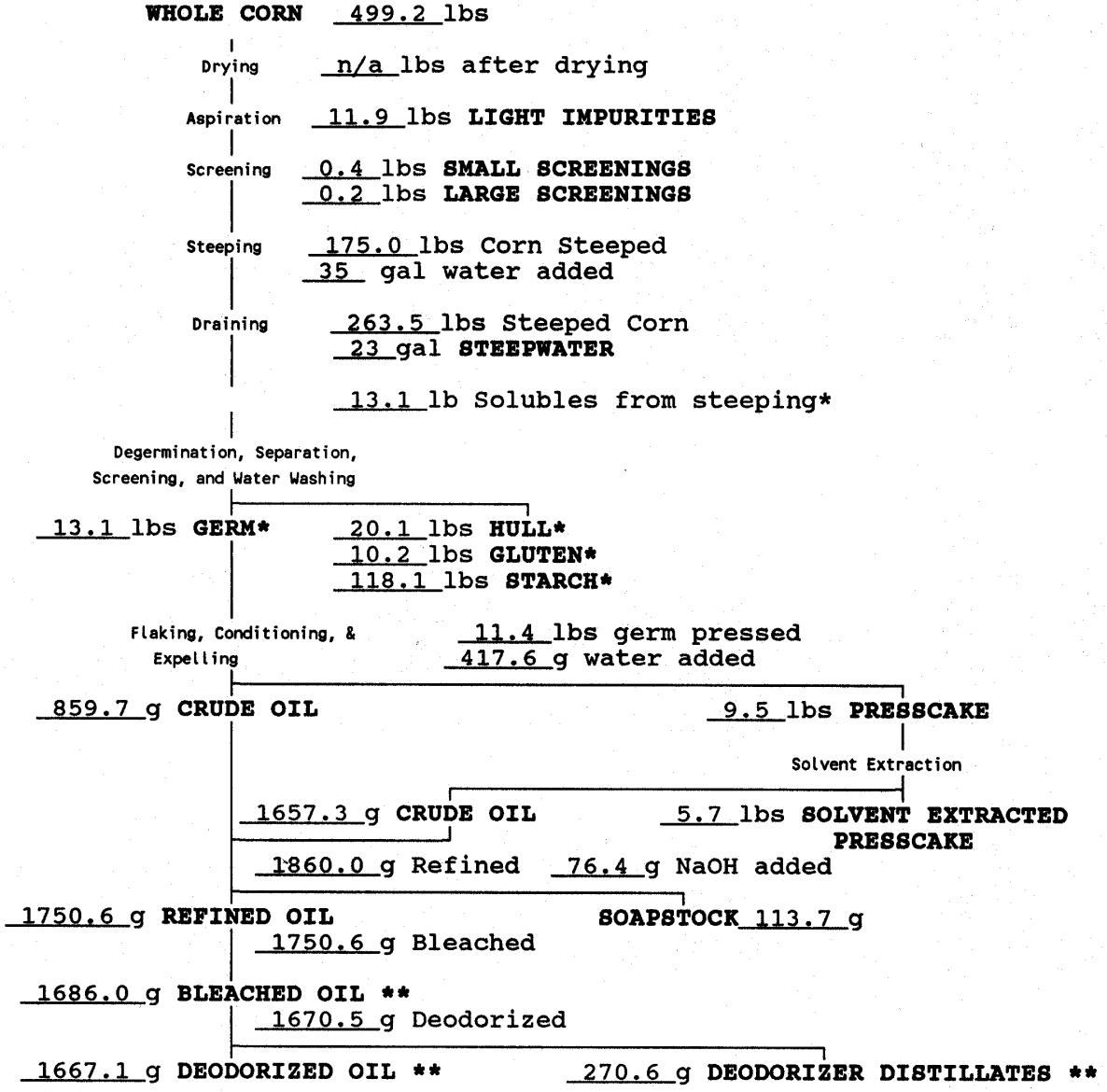
* Calculated amounts based on commercial recovery percentages and starting weight of corn used for wet milling.

** Optional Fractions

REVISION# 05 FORM# 300.5

MATERIAL BALANCE of WET CORN

Sample # **2 (Starlink®)** Code # **CM00B010-03**



* Calculated amounts based on commercial recovery percentages and starting weight of corn used for wet milling.

** Optional Fractions

APPENDIX

Raw Data in support of this study are available under separate cover.

Appendix 9: Detection of transgenic DNA sequences in dry milled fractions, wet milled fractions and masa processed fractions and processed foods made from 100% StarLink™ Grain.

Aventis CropScience

Aventis

Title

Detection of transgenic DNA sequences in dry milled fractions, wet milled fractions and masa processed fractions and processed foods made from 100% StarLink™ Grain

Authors

Kristof Van der Meeren
Marc De Beuckeleer

Completed on

April 9th, 2001

Testing Facility

Aventis CropScience N.V.
Biotech Product Characterization
Jozef Plateaustraat 22
B-9000 Gent
Belgium

Aventis CropScience

Aventis

APPROVALS PAGE

Author **Kristof Van Der Meeren** _____ 9-04-2001
(signature) (date)

Group Leader Molecular Characterization - Biotech Product Characterization

Author **Marc De Beuckeleer** _____ April 9th 2001
(signature) (date)

SUMMARY

This study was undertaken to determine if transgenic CBH351 DNA sequences could be detected in dry milled, wet milled and masa processed fractions and processed foods prepared from grain from a field planted to 100% StarLink™ hybrid corn by small-scale methods. A companion study, utilized these same set of processed fractions and food samples to determine the amount of Cry9C protein that could be detected (Shillito, *et al.,* 2001).

For the purpose of this study, DNA was isolated from non-StarLink™ control and StarLink™ fractions and food samples with the Wizard® DNA extraction protocol. The integrity assessment of the extracted DNA samples showed that the isolated DNA was degraded to variable extends and that no differences in integrity could be observed between the non-StarLink™ control and respective StarLink™ fractions and food samples.

To circumvent limitations caused by strong target DNA degradation, PCR strategies were applied in which small DNA fragments (< 200 bp) were amplified. The PCR analysis was based on the verification of the suitability of the isolated DNA samples for PCR analysis; amplification of two transgenic target sequences; and a specificity assessment of the obtained PCR products.

For the verification of the suitability of the isolated DNA samples for PCR analysis, amplification of endogenous gene sequences was demonstrated. PCR reactions performed with all isolated non-StarLink™ control and StarLink™ samples (except for the refined oil samples) yielded amplicons of the expected size, showing that the quality and quantity of the DNA samples sufficed for PCR analysis. Isolated refined oil templates were fortified with genomic CBH351 DNA and subjected to PCR analysis using primer-pairs to detect P35S-*cry9C* and CBH351 integration sequences. With both primer-pairs specific amplicons were obtained, demonstrating that the refined oil templates used did not contain inhibiting substances. Control refined oil samples were fortified with genomic CBH351 DNA to demonstrate the suitability of the DNA extraction method used. Obtained PCR results demonstrated that DNA, suitable for PCR amplification, could be recovered from the samples.

For all of the non-StarLink™ control samples, presence of the *cry9C* and *bar* transgenic sequences could not be detected, demonstrating that the control grain was not contaminated by any StarLink™ grain.

For all of the StarLink™ fractions and food samples, the presence of the *cry9C* and *bar* transgenic sequences were detected, except for the refined oil samples. The specificity of the obtained transgenic PCR products was demonstrated by restriction enzyme analysis for all fractions.

1. OBJECTIVE

To determine if transgenic sequences are present in dry milled fractions, wet milled fractions and masa processed fractions and processed foods prepared from grain from a field planted to 100% StarLink™ hybrid corn by small-scale methods. A companion study, utilized these same set of processed fractions and food samples to determine the amount of Cry9C protein that could be detected (Shillito, *et al.,* 2001). In that study, the analytical method was the sensitive Cry9C protein ELISA.

2. TEST SUBSTANCES AND CONTROL SUBSTANCES

The test substances were dry milled fractions, wet milled fractions and masa processed fractions and processed foods prepared from StarLink™ field corn grain (hereto referred as StarLink grain, Garst 8600BLT) which was obtained under Aventis CropScience study CM00B010. The grain was sampled by Land O'Lakes Research Farm in Iowa and was assigned the sample number CM00B0010-03. A portion of the grain was shipped to the GLP Processing Program of Texas A&M University.

The control substances were prepared from corn grain (hereto referred as control grain, Pioneer 3751), which was sampled by Qualls Agricultural Laboratory in Washington State from a grain bin located on a nearby farm. Corn containing Bt genes is not normally grown in this area. The control grain was assigned the sample number CM00B010-04. A portion of the grain was shipped to the GLP Processing Program of Texas A&M University.

Table 1: Control and StarLink sample list

Prepared from	Prepared by	Assigned BT-ID	
		Control	StarLink
Product			
Whole grain	From trial locations	454A	455A
Corn meal (dry milled)	Dr. Malcolm Gerngross	459A	459B
Corn flour (dry milled)	Dr. Malcolm Gerngross	461A	461B
Wet milled			
Wet milled starch	Dr. Malcolm Gerngross	454B	455B
Wet milled gluten	Dr. Malcolm Gerngross	454C	455C
Refined oil	Dr. Malcolm Gerngross	457B	457D

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Prepared from	Prepared by	Assigned BT-ID	
		Control	StarLink
Masa process			
Tortillas (soft)	Dr. Lloyd Rooney	418N	414A
Tortillas (fried)	Dr. Triveni Shukla	449A	-
Tortillas (fried)	Dr. Lloyd Rooney	-	414B
Cereals and Snacks			
Puffed cereals	Dr. Triveni Shukla	450A	450C
Corn puffs	Tom Diehl	452A	452B
Corn flakes	Dr. Malcolm Gerngross	453A	453B
Baked goods			
Corn muffins	Debbie Lohmeyer	456E	456H
Corn muffins	Debbie Lohmeyer	456F	456J
Corn bread	Debbie Lohmeyer	456K	456N
Other			
Polenta	Debbie Lohmeyer	456B	456D

3. OVERVIEW OF EXPERIMENTAL DESIGN

DNA was extracted from the different Control and StarLink processed and food samples, using a resin-based DNA extraction protocol. For the verification of the suitability of the isolated DNA for PCR analysis, amplification of endogenous gene sequences was checked. Presence or absence of transgenic sequences was demonstrated using a PCR protocol for the detection of P35S-*cry9C* and CBH351 integration sequences (D-PCR). To determine the specificity of the obtained amplicons, restriction enzyme analysis was performed on the PCR products of both transgenic targets.

4. MOLECULAR ANALYSIS

4.1 Preparation of template DNA

All DNA preparation steps were performed in a PCR Laminar Flow Prep Workstation in a dedicated DNA extraction room in order to prevent cross-contamination. Template preparation of each sample was performed in duplicate.

Purification of DNA was performed based on a protocol developed by Andreas Zimmerman, Jurg Luthy and Urs Pauli (Zeitschrift für Lebensmittel-Untersuchung und-Forschung (1998) 207: 81-90). The samples were crushed in a mortar. Three hundred mg (*) of crushed substance was mixed in a 2 ml Eppendorf microfuge tube with 860 µl 1xTNE buffer [10 mM Tris-HCl pH8, 150 mM NaCl, 2 mM EDTA, 1% SDS], 100 µl guanidinium hydrochloride (3 M) and 40 µl proteïnase-K (20 mg/ml) and then incubated at 55°C for 3 hours. After centrifugation (10 min. at 20000 x g) 500 µl supernatant was combined with 1 ml Wizard® resin (Promega), mixed by inversion and applied on a Wizard® minicolumn using a 5 ml syringe. After washing with 2 ml of 80% isopropanol and drying the minicolumn at room temperature, the DNA was eluted with 75 µl preheated (70°C) water. Finally, to ensure that the DNA samples were not contaminated with RNA, the solutions were treated with 2 µl RNase H (10 mg/ml). The DNA concentration was determined using the Picogreen dsDNA quantification kit (Molecular Probes).

(*) Because the DNA content of refined oil and starch was expected to be minimal, the protocol for DNA extraction from these fractions was adapted. For the extraction of DNA from refined oil and starch, respectively 3 ml and 1000 mg starting material was mixed in a 10 ml Falcon tube with 8.6 ml 1xTNE, 1 ml guanidinium hydrochloride (3 M) and 40 µl proteinase-K (20 mg/ml). After incubation and centrifugation, 4ml of the aqueous phase was mixed with 1ml Wizard® resin and drawn through a Wizard® minicolumn. The DNA was eluted with 75 µl preheated water.

4.2 PCR analysis

Three PCR reactions were performed on each isolated DNA sample. A first PCR reaction, amplifying endogenous gene sequences, was performed to check the suitability of the isolated DNA for PCR analysis. Two PCR reactions were performed to demonstrate the presence/absence of transgenic DNA sequences (P35S-*cry9C* and CBH351 D-PCR).

4.2.1 Primers and amplified fragments

Table 2: PCR primers

Primer	Sequence (5' → 3')(*)	Description
CVZ16	CgC.CTT.TCC.AgC.ATC.AAT.gTC.g	Sense primer *aldolase* gene (**)
DPA121	CCC.TCC.TTg.Agg.ACA.TCA.AC	Anti-sense primer *aldolase* gene (**)
MDB498	TAT.CCT.TCg.CAA.gAC.CCT.TCC	Sense primer P35S promotor
MDB497	ATg.TAg.CTg.TCg.gTg.TAg.TCC	Anti-sense primer *cry9C* gene
DPA18	gCg.gTg.TCA.TCT.ATg.TTA.CTA.g	sense primer 3'nos
DPA123	TCT.gCC.CAT.Cgg.AgT.TAT.TTC.C	Anti-sense primer CBH351 plant DNA

(*): To avoid confusion between bases, a lower-case 'g' is used to clearly differentiate between 'g' and 'C'.

(**): Database accession number M16220 (The complete amino acid sequence for the anaerobically induced aldolase from maize derived from cDNA clones).

Table 3: amplified fragments

Target	Primer-pair	Amplicon size	Specificty assessment
aldolase gene	CVZ16 – DPA121	172bp	Not relevant
P35S-*cry9C*	MDB497 – MDB498	175bp	*MseI* digest (*) 115 bp + 62 bp
CBH351 D-PCR	DPA18 – DPA123	178bp	*HhaI* digest (**) 104 bp + 76 bp

(*): *MseI* recognition site: T↓TAA. Both restriction fragments will carry an 'AT' overhang. The actual sum of the size of the obtained restriction fragments will therefor be larger then the amplicon size (177 bp instead of 175 bp).

(**): *HhaI* recognition site: gCg↓C. Both restriction fragments will carry a 'Cg' overhang. The actual sum of the size of the obtained restriction fragments will therefor be larger then the amplicon size (180 bp instead of 178 bp).

4.2.2 PCR conditions

To set up the PCR, a Master Mix containing all components was prepared in a dedicated Master Mix set-up room. The Master Mix was prepared for a number of PCR's, which was greater than the number of PCR's actually required, in order to account for residual volume in the microfuge tube. The thawed components were maintained on ice while preparing the Master Mix. *AmpliTaq Gold* DNA polymerase was added just before dispensing the appropriate volume of the Master Mix into the reaction vessels. Adding the Master Mix to the template DNA was performed in a second PCR workstation in order to prevent cross-contamination.

Composition Master Mixes (volumes for one reaction):

a) Endogenous control target

2.5 µl 10X PCR buffer (Pharmacia)
1.5 µl 25 mM $MgCl_2$
0.5 µl dNTP's (10 mM)
1 µl CVZ16 (10 pmol/µl)
1 µl DPA121 (10 pmol/µl)
0.1 µl *AmpliTaq Gold* DNA-polymerase (5 U/µl)
16.4 µl water

23 µl of the Master Mix was added to 2 µl template DNA (5ng/µl) (*).

b) P35S-*cry9C* target

2.5 µl 10X PCR buffer (Pharmacia)
1.5 µl 25 mM $MgCl_2$
0.5 µl dNTP's (10 mM)

1 µl MDB497 (10 pmol/µl)
1 µl MDB498 (10 pmol/µl)
0.1 µl *AmpliTaq Gold* DNA-polymerase (5 U/µl)
16.4 µl water

23 µl of the Master Mix was added to 2 µl template DNA (5 ng/µl) (*).

c) CBH351 border sequence target (CBH351 D-PCR)

2.5 µl 10X PCR buffer (Pharmacia)
1.5 µl 25 mM $MgCl_2$
0.5 µl dNTP's (10 mM)
1 µl DPA18 (10 pmol/µl)
1 µl DPA123 (10 pmol/µl)
0.1 µl *AmpliTaq Gold* DNA-polymerase (5 U/µl)
16.4 µl water

23 µl of the Master Mix was added to 2 µl template DNA (5 ng/µl) (*).

(*): The starch and refined oil fractions contained undetectable amounts of template DNA. Two µl of template solution was used.

4.2.3 PCR thermocycling profile

Thermocycler: MJ Research PTC-200

	10 min. at 95°C
Followed by:	1 min. at 95°C 1 min. at 64°C 2 min. at 72°C For 5 cycles
Followed by:	30 sec. at 95°C 30 sec. at 64°C 1 min. at 72°C For 30 cycles
Followed by:	10 min. at 72°C

The PCR thermocycling profiles for all three PCR reactions were identical.

4.2.4 Specificity assessment

In order to determine the specificity of the obtained amplicons, restriction enzyme analysis was performed on the PCR products of the P35S-*cry9C* and the CBH351 D-PCR reactions using respectively the *MseI* and *HhaI* restriction enzymes (see Table 3). Ten µl of PCR product were digested by adding 5 units restriction enzyme, applying buffer and temperature according to conditions proposed by the manufacturer. The digests were incubated overnight at 37°C. Two µl of gel-loading dye were added and the digests were stored frozen until loading of the gel.
Each digest was loaded in the appropriate sample well of a 4% agarose gel. A 20 bp ladder (Biorad) was used as a molecular weight. Electrophoresis was carried out at 2 to 4 V/cm until the bromophenol blue dye had migrated approximately 12 cm.

4.2.5 Visualization of the PCR and restriction products

A 4% agarose gel was prepared by combining 12 g of agarose MP (Boehringer Mannheim) with 300 ml of 1×TBE gel running buffer (1×TBE: 0.09 M Tris-borate, 0.002 M EDTA, pH 8.0) in a 500 ml bottle. 12 µl of a 10mg/ml ethidium bromide solution was added. The gel mixture was heated in a microwave oven, stopping to swirl the contents of the bottle occasionally. The mixture was cooled down to approximately 65°C. The gel was cast in a submarine horizontal gel support on which a 40 toothcomb was placed. The toothcomb was removed from the solidified gel and the gel was placed in an electrophoresis tank containing 1×TBE gel running buffer.

Three µl loading dye was added to 10 µl of each PCR reaction and restriction enzyme reaction. The samples were loaded on the prepared 4% agarose gel and electrophoresis was carried out at 2 to 4 volts/cm.

To photograph the gel it was carefully transferred to the UV transilluminator. The image was acquired, processed and printed on paper using the Imagemaster Video Documentation System from Amersham Pharmacia Biotech).

4.2.6 Assigned positive and negative controls to a PCR run

Every PCR run includes control samples to validate the PCR results. Data from DNA samples within a single PCR run and a single PCR Master Mix will not be acceptable unless all control samples show the expected results:

- A DNA positive control (POS): this is a PCR in which the template DNA provided is 10 ng genomic DNA prepared from a *Zea mays* plant containing the CBH351 elite event. Successful amplification of this control demonstrates that the PCR was performed under conditions that allowed satisfactory amplification of the transgenic target sequences.

- The DNA negative control (NEG): this is a PCR on a water sample on which all template preparation steps were performed. When the expected result (no PCR products) is observed, this indicates that the template preparation components are not contaminated with target DNA.

- The No Cross-contamination Control (NCC): this is a PCR on a water sample, which was placed into the workstation during template preparation of the fractions. When the expected result is observed (no PCR products), this indicates that obtained PCR products are not obtained through DNA cross-contamination during template preparation.

- The No Template Control (NTC): this is a PCR in which sterile milli-Q water was added to the Master Mix. When the expected result (no PCR product) is observed, this indicates that the PCR Master Mix is not contaminated with target DNA.

5. RESULTS AND DISCUSSION

PCR-based detection methods were applied to samples derived from dry milling, wet milling and processed foods, to investigate the presence/absence of transgenic StarLink DNA sequences. The strategy of the analysis was based on following steps:

- extraction of total genomic DNA
- verification of the suitability of the isolated DNA for PCR analysis

- amplification of transgenic target sequences
- specificity assessment of the PCR products

5.1 Preparation of template DNA

All template preparation steps were performed in a PCR workstation (PCR Laminar Flow Prep Station) in a dedicated DNA extraction room. Template preparations of all Control and StarLink processed and food samples were performed in duplicate. Three hundred mg of the substances was used to isolate genomic DNA (except for refined oil: 3 ml; and the starch: 1000 mg). The concentration of each DNA preparation was measured using the PicoGreen® dsDNA quantitation kit from Molecular Probes. Obtained values are summarized in Table 4 (see annex I).

The quality and efficiency of DNA extractions of all substances were checked using agarose gel electrophoresis. Approximately 100 ng template was loaded on a 1.5% agarose gel and electrophoresis was carried out at 2 to 4 volts/cm.

The integrity assessment of the extracted DNA samples showed that the isolated DNA was degraded to a variable extend (see Figure 1). Predominantly high-molecular-weight DNA was isolated from the whole grain, corn meal and corn flour Control and StarLink samples (see Figure 1, lanes 1 through 12). The extracted DNA from gluten, tortillas (soft), tortillas (fried), corn muffins, corn bread and polenta was degraded, resulting in fragment sizes ranging between about 100 bp to 10 Kb (see Figure 1, lanes 17 through 20; lanes 31 through 38; lanes 51 through 66). DNA isolated from puffed cereals, corn puffs and corn flakes was highly degraded and had an average fragment size of about 300 bp (see Figure 1, lanes 30 through 50). The obtained results show that there are no differences in integrity between the Control and respective StarLink samples.

DNA extraction from starch and refined oil samples didn't yield measurable DNA quantities (see Table 4 and Figure 1, lanes 13 through 16).

The variability in DNA yield was for most of the substances very low. For gluten samples we observed a greater variability (approximately ten fold) in DNA yield between the Control and the StarLink samples. This variability was confirmed in a second series of DNA extractions from gluten samples.

5.2 PCR analysis

The PCR analysis included control samples to validate the PCR results. Data from DNA samples within a single PCR run and a single PCR Master Mix were not acceptable unless all control samples showed the expected results. With all Master Mixes and all PCR runs the expected results were obtained. Control and corresponding StarLink DNA templates were used in the same PCR run, using the same Master Mix.

PCR analysis results of all Control and StarLink samples are summarized in Tables 5 through 18 (see annex II). Examples of gel agarose analysis of endogenous, P35S-*cry9C* and CBH351 D-PCR target detection are presented in Figures 2 through 44 (see annex II).

To circumvent limitations caused by a strong degradation of isolated target DNA, we used detection strategies in which small DNA fragments (< 200 bp) are amplified (see Table 3).

5.2.1. Endogenous control target

Substances present in the different matrixes can be co-extracted with the DNA, and thus interfere with the PCR (inhibition). In order to control the suitability of the isolated DNA for PCR analysis, amplification of endogenous gene sequences was checked.

PCR reactions performed with all isolated DNA samples (except for the refined oil samples) yielded amplicons of the expected size (172 bp). These results showed that the quality of the DNA samples did allow for a PCR product to be generated. Furthermore, this result confirmed that the content of *Zea mays* DNA within the total isolated DNA from the processed food samples was sufficiently high to permit PCR amplification. The endogenous control PCR yielded almost equal concentrations of amplification products (as judged by eye) in all cases, indicating that the concentration of potential PCR inhibitors was not a relevant factor.

The DNA concentration of the refined oil templates could not be measured (see Table 4) and PCR reactions performed with these templates did not yield any PCR product (see Figure 17). To exclude the presence of PCR inhibitors in the isolated templates, additional experiments were performed.

Isolated refined oil templates and water samples, were fortified with different amounts of total genomic CBH351 DNA (ranging from 10 ng to 0.001 ng). The fortified samples were subjected to PCR analysis, using the primer-pairs targeting the CBH351 flanking sequences and the P35S-*cry9C* target. As shown in Figure 18, the isolated refined oil template fortified with 0.1 ng total genomic CBH351 DNA still yielded a specific visible 175 bp P35S-*cry9C* fragment. With the fortified water samples we observed the same sensitivity. When using the CBH351 integration specific primer-pair, a specific 178 bp fragment was visible with refined oil template DNA fortified with 0.01 ng total genomic CBH351 DNA. These results demonstrate that the template used for the PCR analysis did not contain inhibiting substances.

To demonstrate that the DNA extraction method used was adequate to isolate DNA (when present) from refined oil samples a second fortification experiment was performed. Three ml refined oil Control samples were fortified with different amounts (ranging from 0 ng through 50 ng) of genomic CBH351 DNA before DNA extraction. Total DNA was extracted and eluted from the Wizard® minicolumn using 75 µl preheated water. Two µl template was used in a PCR reaction targeting the CBH351 integration fragment and the P35S-*cry9C* sequence. As shown in Figures 19 and 20, three ml refined oil sample fortified with 2.5 ng total genomic CH351 DNA yielded a visible specific 178 bp CBH351 integration fragment, and a 3 ml refined oil sample fortified with 5 ng total genomic CBH351 DNA yielded a visible specific 175 bp P35S-*cry9C* fragment. This analysis shows that DNA (when present) suitable for PCR amplification can be recovered from refined oil samples with the DNA extraction method used.

5.2.2. P35S-*cry9C* and CBH351 D-PCR targets

Scoring of the results with the transgenic primer-pairs was performed according to following rules:

- Lanes showing visible amounts of the PCR product of the expected size, and of the restriction fragments of the expected size, indicate that the corresponding sample from which the template DNA was prepared, contains the sequence assayed for.

- Lanes not showing visible amounts of the PCR product, indicate that the corresponding sample from which the template DNA was prepared, doesn't contain the sequence assayed for.

All control samples were shown to be negative for the presence of P35S-*cry9C* and CBH351 integration sequences. All corresponding StarLink samples (except for the refined oil: samples not tested (see 5.2.1.)) were shown to contain the P35S-*cry9C* and CBH351 integration sequences. The specificity of the amplified fragments was always demonstrated by restriction digestion analysis.

To investigate the presence of transgenic sequences in, for instance, wet milled starch, the primer-pair DPA18-DPA123 was applied in the PCR permitting the specific detection of CBH351 integration sequences. As shown in Figure 12, no amplification products were obtained with the DNA templates isolated from starch Control samples (lanes 1a, 1b, 2a and 2b). With DNA templates isolated from starch StarLink samples a 178 bp amplicon was obtained as the exclusive PCR product (lanes 3a, 3b, 4a and 4b). The specificity of the PCR was confirmed by restriction digestion of the obtained amplicons, using the *HhaI* restriction enzyme. As shown in Figure 12, all amplicons yielded two restriction fragments with the expected 104 bp and 76 bp sizes (see lanes 3a', 3b', 4a' and 4b'). A second PCR was performed for the detection of P35S-*cry9C* sequences, using primer-pair MDB497-MDB498. As shown in Figure 13, no amplification products were obtained with the DNA templates isolated from the starch Control samples (lanes 1a, 1b, 2a and 2b). The expected 175 bp amplicon was obtained as the exclusive PCR product with DNA templates isolated from starch StarLink samples (see lanes 3a, 3b, 4a and 4b). Restriction enzyme analysis of the obtained amplicons, using the *MseI* restriction enzyme, yielded two restriction fragments with the expected 115 bp and 62 bp sizes (see lanes 3a', 3b', 4a' and 4b').

Partial digestion of the obtained amplicons was sometimes observed with both *MseI* and *HhaI* restriction digests (for example see Figure 7, lanes 3a', 3b', 4a' and 4b'). Next to the expected 115bp and 62 bp *MseI* fragments, a 175 bp fragment could be observed. This 175 bp fragment represents the undigested amplicon.

6. CONCLUSIONS

Integrity assessment of the extracted DNA showed that size of the isolated DNA varied from fraction to fraction. The obtained results showed that there are no differences in integrity between the Control and respective StarLink samples.

Suitability of the isolated DNA for PCR amplification was confirmed by amplification of endogenous gene sequences.

For all control samples, presence of the *cry9C* and *bar* transgenic sequences could not be detected.

For all StarLink samples, presence of the *cry9C* and *bar* transgenic sequences was detected, except for the refined oil samples. Fortification of the refined oil samples, with total genomic CBH351 DNA, showed that the DNA extraction method used was adequate and that PCR inhibitors were not present.

Annex I - Concentration and integrity assessment of isolated DNA samples

Table 4: Summary of template preparations of Control and StarLink™ samples.

N°(*)	Product name	BT-ID	Amount starting material	Total DNA concentration (ng/µl)
1	Whole grain	454A	300mg	150.28
2	Whole grain	454A	300mg	126.88
3	Whole grain	455A	300mg	168.32
4	Whole grain	455A	300mg	80.53
5	Corn meal	459A	300mg	95.04
6	Corn meal	459A	300mg	94.73
7	Corn meal	459B	300mg	73.90
8	Corn meal	459B	300mg	77.51
9	Corn flour	461A	300mg	63.24
10	Corn flour	461A	300mg	54.78
11	Corn flour	461B	300mg	75.92
12	Corn flour	461B	100mg	64.53
13	Starch	454B	1000mg	not measurable
14	Starch	454B	1000mg	not measurable
15	Starch	455B	1000mg	not measurable
16	Starch	455B	1000mg	not measurable
17	Gluten	454C	300mg	127.68
18	Gluten	454C	300mg	139.20
19	Gluten	455C	300mg	9.52
20	Gluten	455C	300mg	8.87
21	Refined oil	457B	3ml	not measurable
22	Refined oil	457B	3ml	not measurable
23	Refined oil	457D	3ml	not measurable
24	Refined oil	457D	3ml	not measurable
31	Tortillas (soft)	418N	300mg	26.71
32	Tortillas (soft)	418N	300mg	25.35
33	Tortillas (soft)	414A	300mg	20.79
34	Tortillas (soft)	414A	300mg	19.90
35	Tortillas (fried)	418M	300mg	24.38
36	Tortillas (fried)	418M	300mg	26.39

N°(*)	Product name	BT-ID	Amount starting material	Total DNA concentration (ng/µl)
37	Tortillas (fried)	414B	300mg	26.59
38	Tortillas (fried)	414B	300mg	31.24
39	Puffed cereals	450A	300mg	20.39
40	Puffed cereals	450A	300mg	23.18
41	Puffed cereals	450C	300mg	19.65
42	Puffed cereals	450C	300mg	24.45
43	Corn puffs	452A	300mg	28.87
44	Corn puffs	452A	300mg	28.81
45	Corn puffs	452B	300mg	21.34
46	Corn puffs	452B	300mg	17.60
47	Corn flakes	453A	300mg	29.20
48	Corn flakes	453A	300mg	21.51
49	Corn flakes	453B	300mg	9.30
50	Corn flakes	453B	300mg	7.44
51	Corn muffins	456E	300mg	10.28
52	Corn muffins	456E	300mg	11.74
53	Corn muffins	456H	300mg	52.31
54	Corn muffins	456H	300mg	37.98
55	Corn muffins	456F	300mg	37.29
56	Corn muffins	456F	300mg	42.75
57	Corn muffins	456J	300mg	13.10
58	Corn muffins	456J	300mg	13.20
59	Corn bread	456K	300mg	29.05
60	Corn bread	456K	300mg	31.48
61	Corn bread	456N	300mg	50.73
62	Corn bread	456N	300mg	51.95
63	Polenta	456B	300mg	7.00
64	Polenta	456B	300mg	8.25
65	Polenta	456D	300mg	11.75
66	Polenta	456D	300mg	12.13

(*): Loading sequence of agarose gel (see Figure 1)

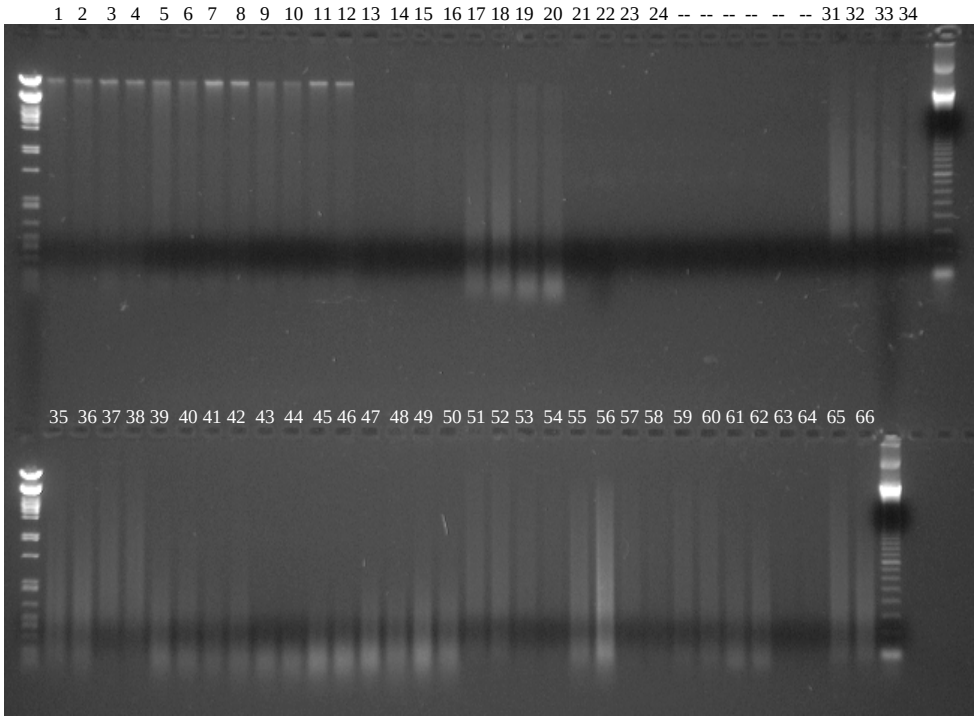

Figure 1: DNA integrity assessment of Control and StarLink™ samples.
Approximately 100 ng template was added to water and 2 µl loading dye and loaded on a 1.5% agarose gel. Electrophoresis was carried out at 2 to 4 volts/cm. Loading sequence of the gel according to Table 4.

Annex II - Summary of PCR analysis

WHOLE GRAIN

Table 5: Whole grain

N(*)	BT-ID	ENDO	P35S-*cry9C*	CBH351 D-PCR
1a	454A (Control sample)	yes	no	no
1b	454A (Control sample)	yes	no	no
2a	454A (Control sample)	yes	no	no
2b	454A (Control sample)	yes	no	no
3a	455A (StarLink™ sample)	yes	yes	yes
3b	455A (StarLink™ sample)	yes	yes	yes
4a	455A (StarLink™ sample)	yes	yes	yes
4b	455A (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 2, 3 and 4.

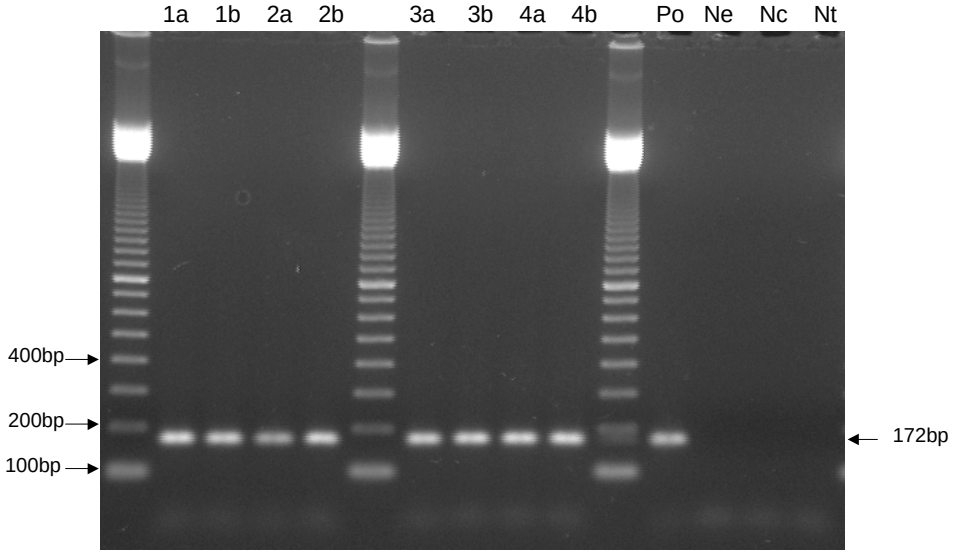

Figure 2: Agarose gel analysis - Endogenous target in whole grain Control and StarLink™ samples. Loading sequence of the gel according to Table 5.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

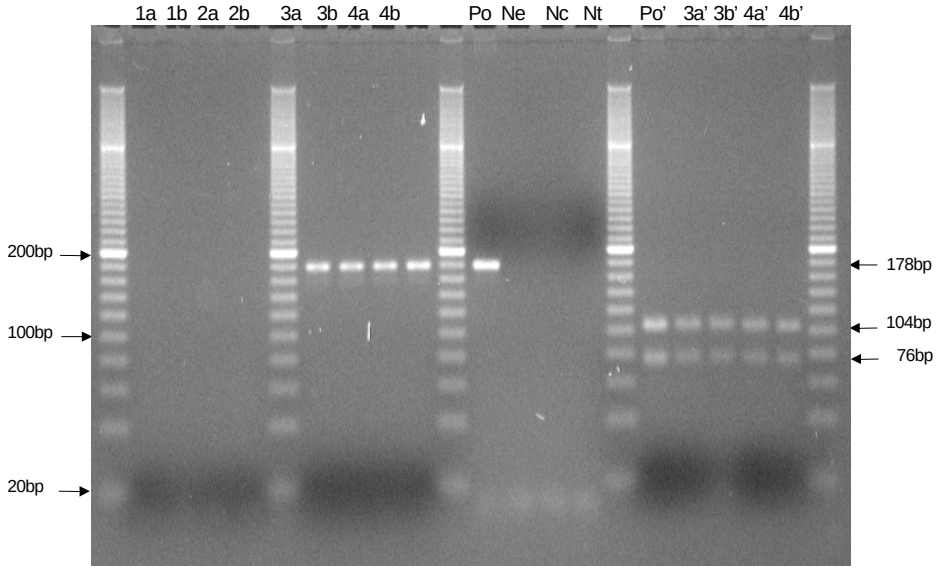

Figure 3: Agarose gel analysis - CBH351 D-PCR target in whole grain Control and StarLink™ samples. Loading sequence of the gel according to Table 5.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

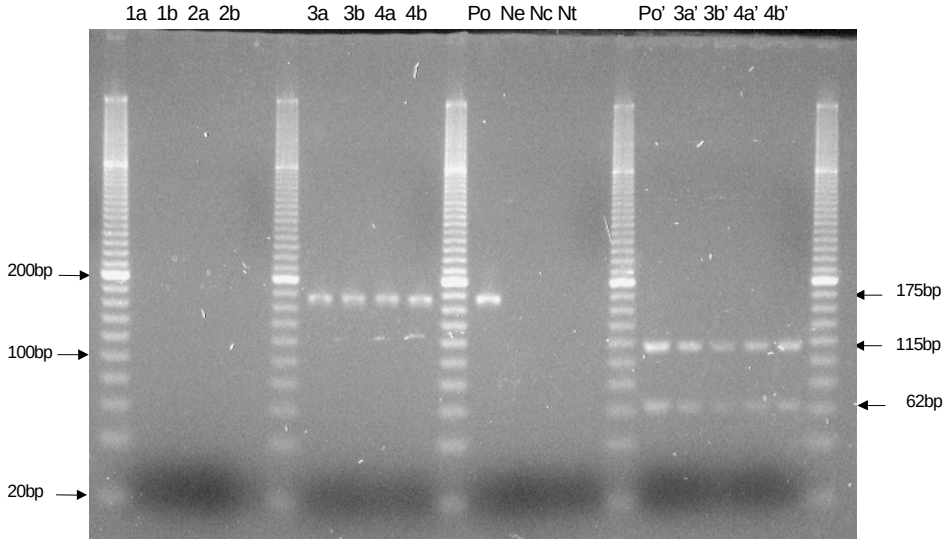

Figure 4: Agarose gel analysis - P35S-*cry9C* target in whole grain Control and StarLink™ samples. Loading sequence of the gel according to Table 5.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

DRY MILLED MEAL

Table 6: Dry Milled Meal fraction

N(*)	BT-ID	ENDO	P35S-*cry9C*	CBH351 D-PCR
1a	459A (Control sample)	yes	no	no
1b	459A (Control sample)	yes	no	no
2a	459A (Control sample)	yes	no	no
2b	459A (Control sample)	yes	no	no
3a	459B (StarLink™ sample)	yes	yes	yes
3b	459B (StarLink™ sample)	yes	yes	yes
4a	459B (StarLink™ sample)	yes	yes	yes
4b	459B (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 5, 6 and 7.

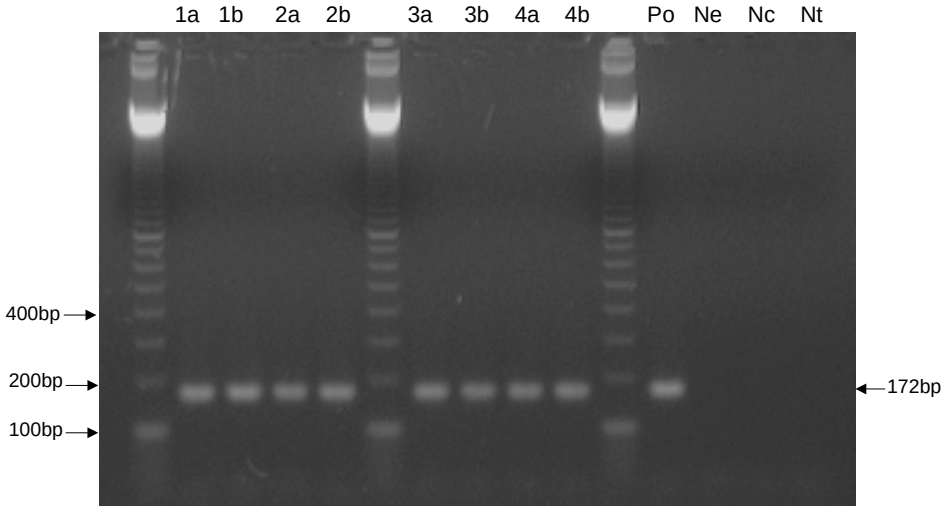

Figure 5: Agarose gel analysis - Endogenous target in corn meal Control and StarLink™ samples. Loading sequence of the gel according to Table 6.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

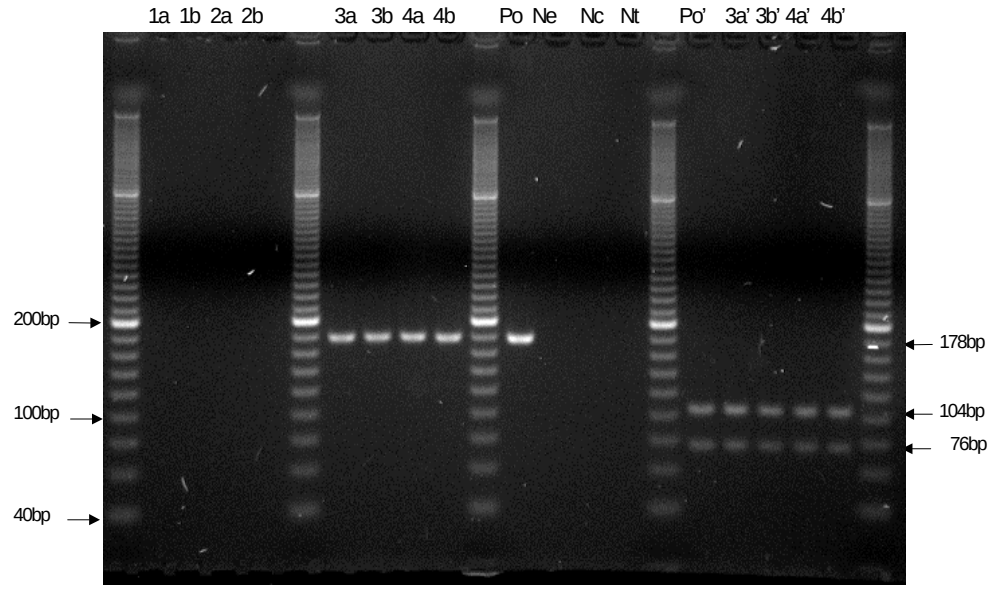

Figure 6: Agarose gel analysis - CBH351 D-PCR target in corn meal Control and StarLink™ samples. Loading sequence of the gel according to Table 6.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

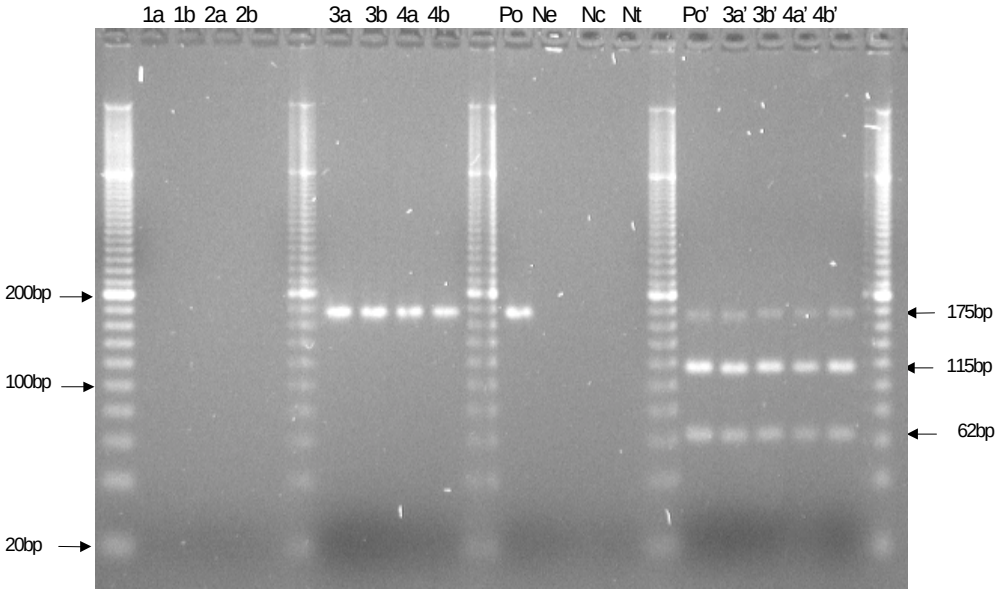

Figure 7: Agarose gel analysis - P35S-*cry9C* target in corn meal Control and StarLink™ samples. Loading sequence of the gel according to Table 6.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

DRY MILLED FLOUR

Table 7: Dry Milled Flour fraction

N(*)	BT-ID	ENDO	P35S-*cry9C*	CBH351 D-PCR
1a	461A (Control sample)	yes	no	no
1b	461A (Control sample)	yes	no	no
2a	461A (Control sample)	yes	no	no
2b	461A (Control sample)	yes	no	no
3a	461B (StarLink™ sample)	yes	yes	yes
3b	461B (StarLink™ sample)	yes	yes	yes
4a	461B (StarLink™ sample)	yes	yes	yes
4b	461B (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 8, 9 and 10.

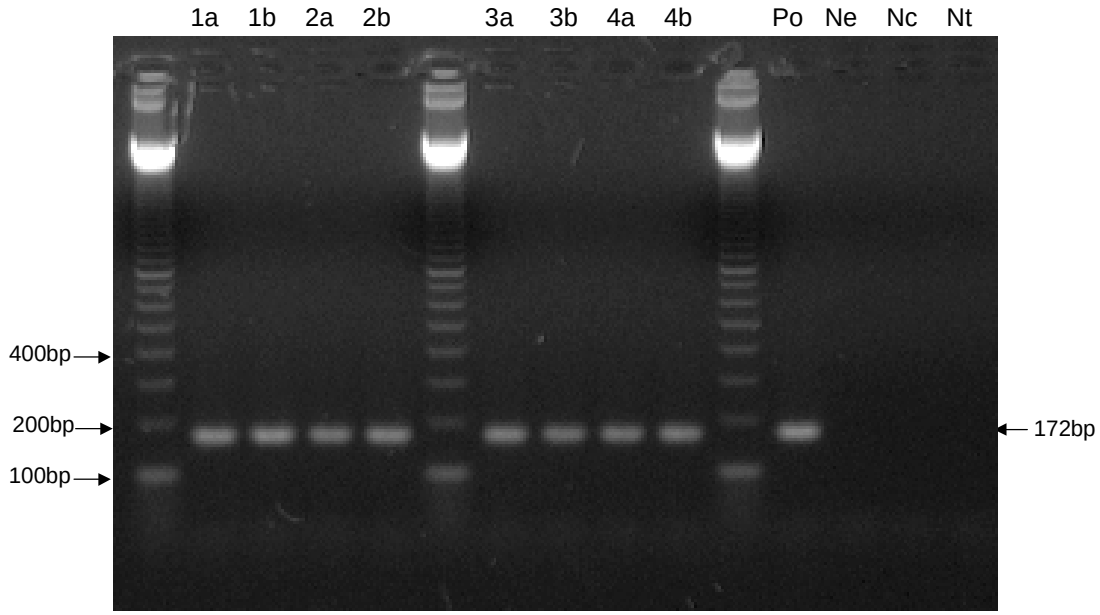

Figure 8: Agarose gel analysis - Endogenous target in corn flour Control and StarLink™ samples. Loading sequence of the gel according to Table 7.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

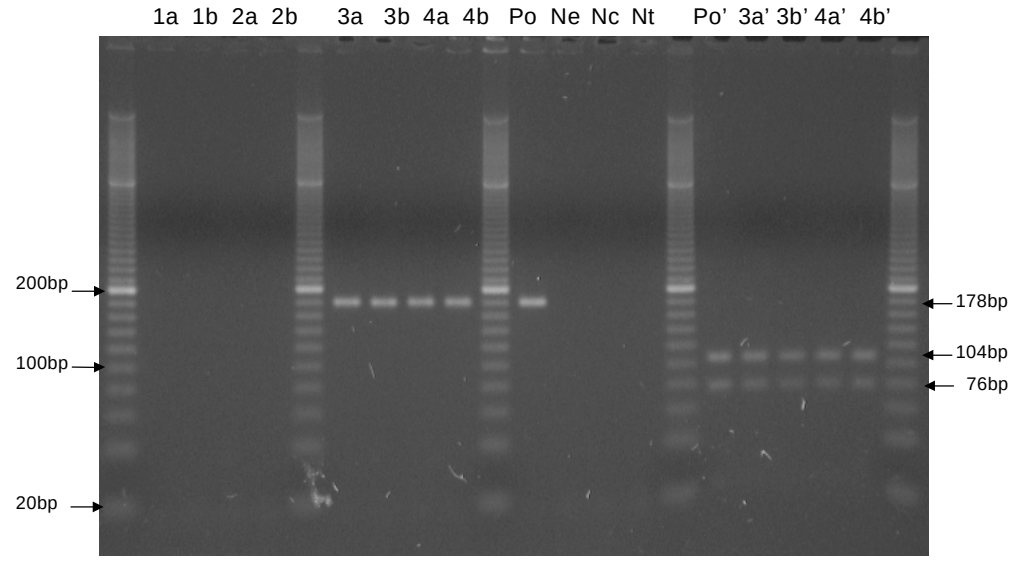

Figure 9: Agarose gel analysis - CBH351 D-PCR target in corn flour Control and StarLink™ samples. Loading sequence of the gel according to Table 7.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

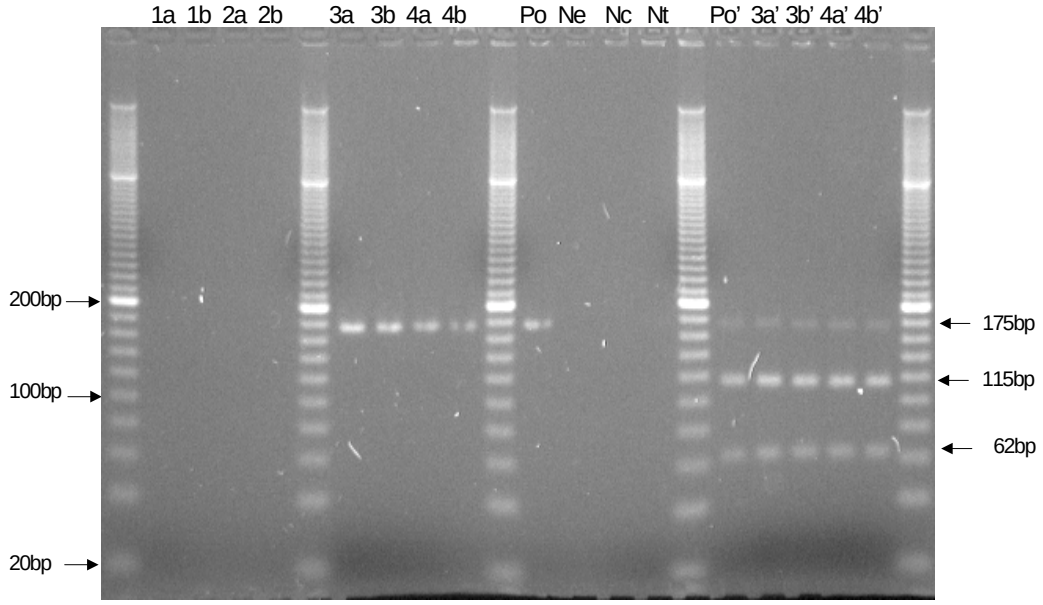

Figure 10: Agarose gel analysis - P35S-*cry9C* target in corn flour Control and StarLink™ samples. Loading sequence of the gel according to Table 7.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

WET MILLED STARCH

Table 8: Wet Milled Starch fraction

N(*)	BT-ID	ENDO	P35S-*cry9C*	CBH351 D-PCR
1a	454B (Control sample)	yes	no	no
1b	454B (Control sample)	yes	no	no
2a	454B (Control sample)	yes	no	no
2b	454B (Control sample)	yes	no	no
3a	455B (StarLink™ sample)	yes	yes	yes
3b	455B (StarLink™ sample)	yes	yes	yes
4a	455B (StarLink™ sample)	yes	yes	yes
4b	455B (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 11, 12 and 13.

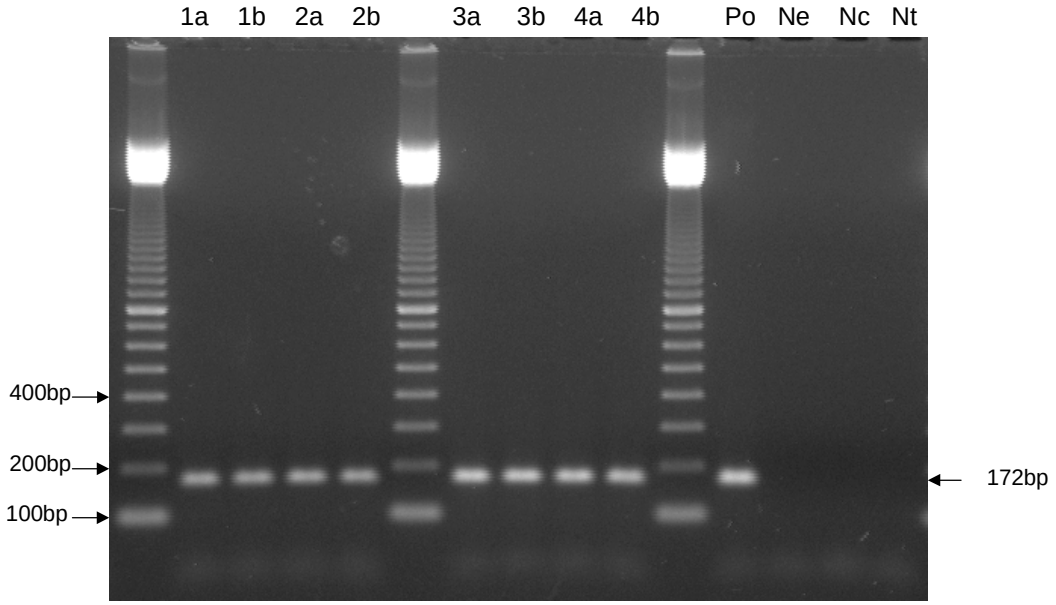

Figure 11: Agarose gel analysis - Endogenous target in starch Control and StarLink™ samples. Loading sequence of the gel according to Table 8.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

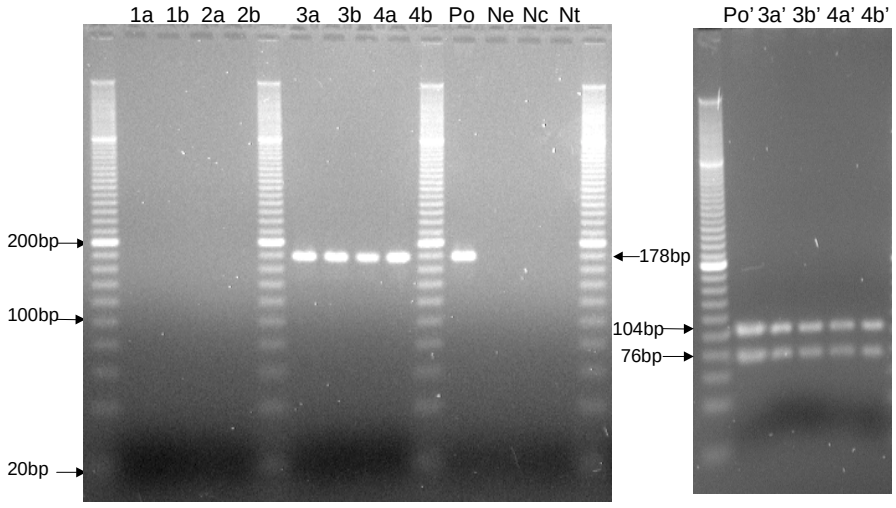

Figure 12: Agarose gel analysis - CBH351 D-PCR target in starch Control and StarLink™ samples. Loading sequence of the gel according to Table 8.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

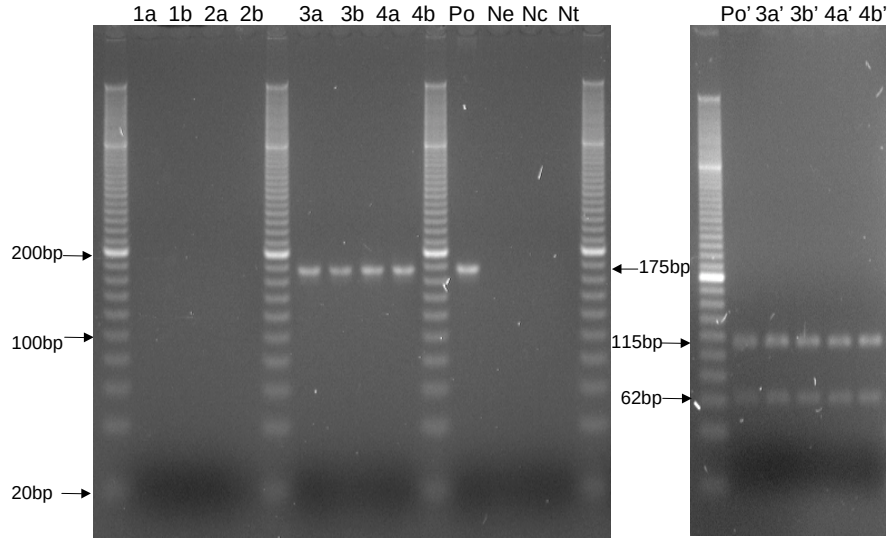

Figure 13: Agarose gel analysis - P35S-*cry9C* target in starch Control and StarLink™ samples. Loading sequence of the gel according to Table 8.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

WET MILLED GLUTEN

Table 9: Wet Milled Gluten fraction

N(*)	BT-ID	ENDO	P35S-*cry9C*	CBH351 D-PCR
1a	454C (Control sample)	yes	no	no
1b	454C (Control sample)	yes	no	no
2a	454C (Control sample)	yes	no	no
2b	454C (Control sample)	yes	no	no
3a	455C (StarLink™ sample)	yes	yes	yes
3b	455C (StarLink™ sample)	yes	yes	yes
4a	455C (StarLink™ sample)	yes	yes	yes
4b	455C (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 14, 15 and 16.

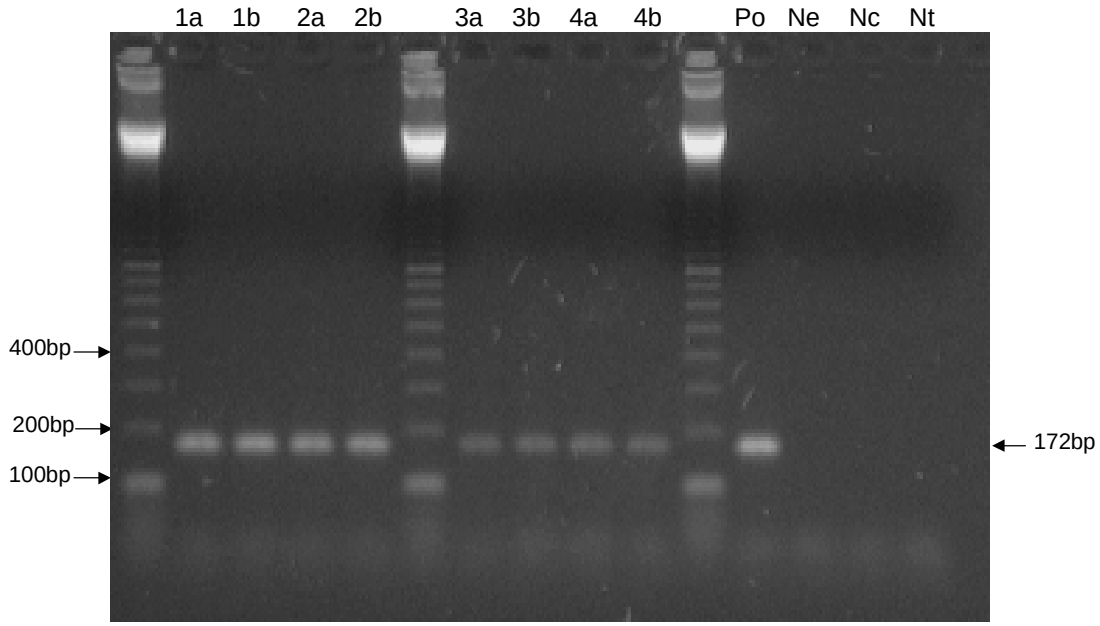

Figure 14: Agarose gel analysis - Endogenous target in gluten Control and StarLink™ samples. Loading sequence of the gel according to Table 9.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

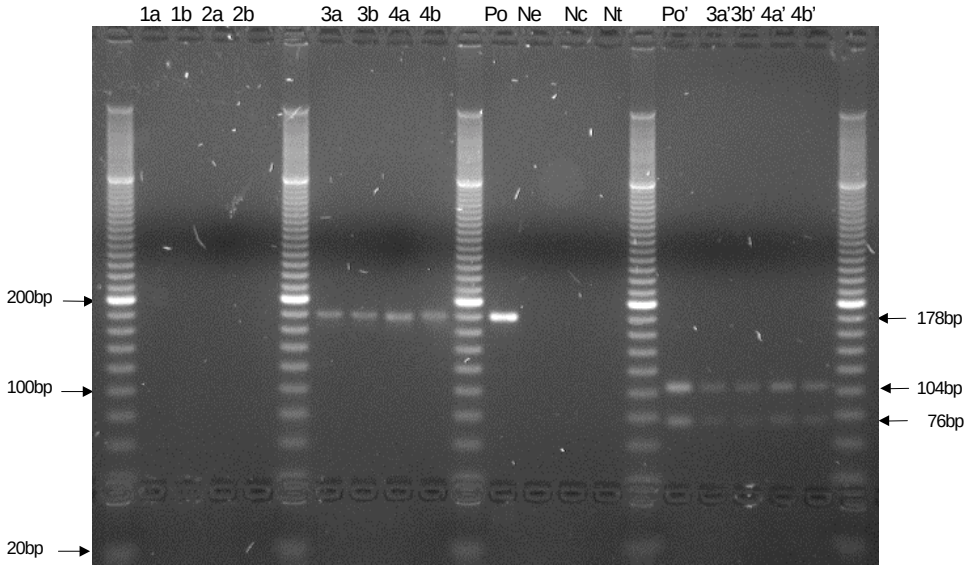

Figure 15: Agarose gel analysis - CBH351 D-PCR target in gluten Control and StarLink™ samples. Loading sequence of the gel according to Table 9.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

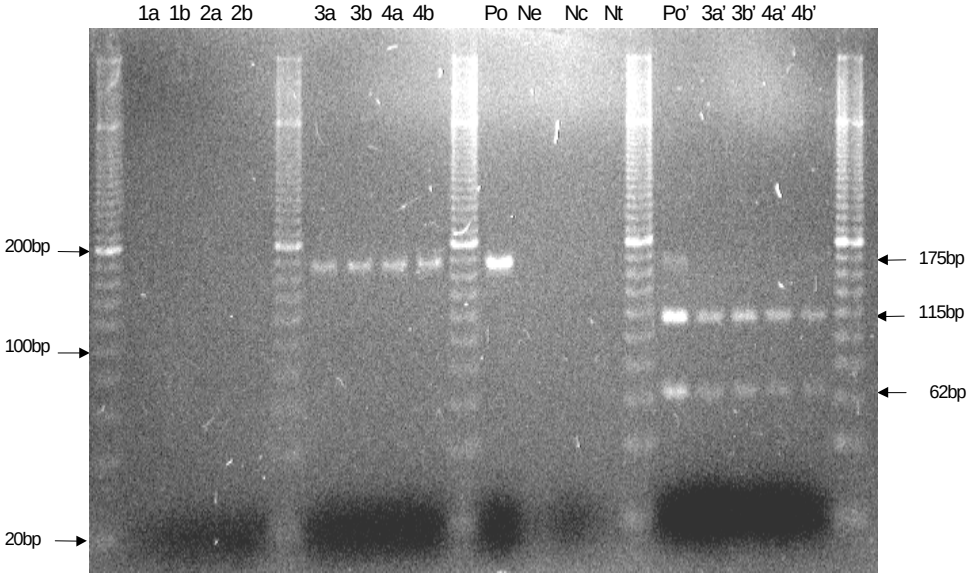

Figure 16: Agarose gel analysis - P35S-*cry9C* target in gluten Control and StarLink™ samples. Loading sequence of the gel according to Table 9.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

REFINED OIL

Table 10: Refined Oil fraction

N(*)	BT-ID	ENDO	P35S-*cry9C*	CBH351 D-PCR
1a	457B (Control sample)	no	Not tested	Not tested
1b	457B (Control sample)	no	Not tested	Not tested
2a	457B (Control sample)	no	Not tested	Not tested
2b	457B (Control sample)	no	Not tested	Not tested
3a	457D (StarLink ™ sample)	no	Not tested	Not tested
3b	457D (StarLink ™ sample)	no	Not tested	Not tested
4a	457D (StarLink ™ sample)	no	Not tested	Not tested
4b	457D (StarLink ™ sample)	no	Not tested	Not tested

(*) Loading sequence of Figure 17

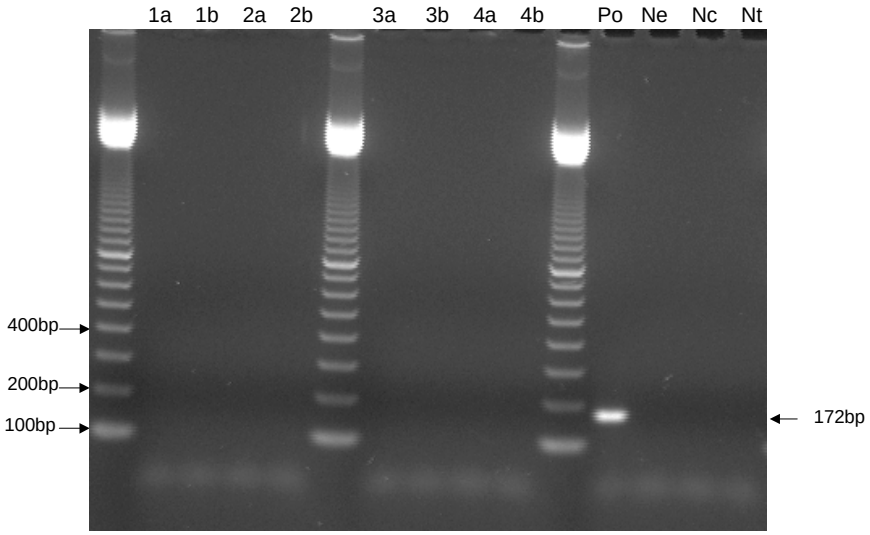

Figure 17: Agarose gel analysis - Endogenous target in refined oil Control and StarLink™ samples. Loading sequence of the gel according to Table 10.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

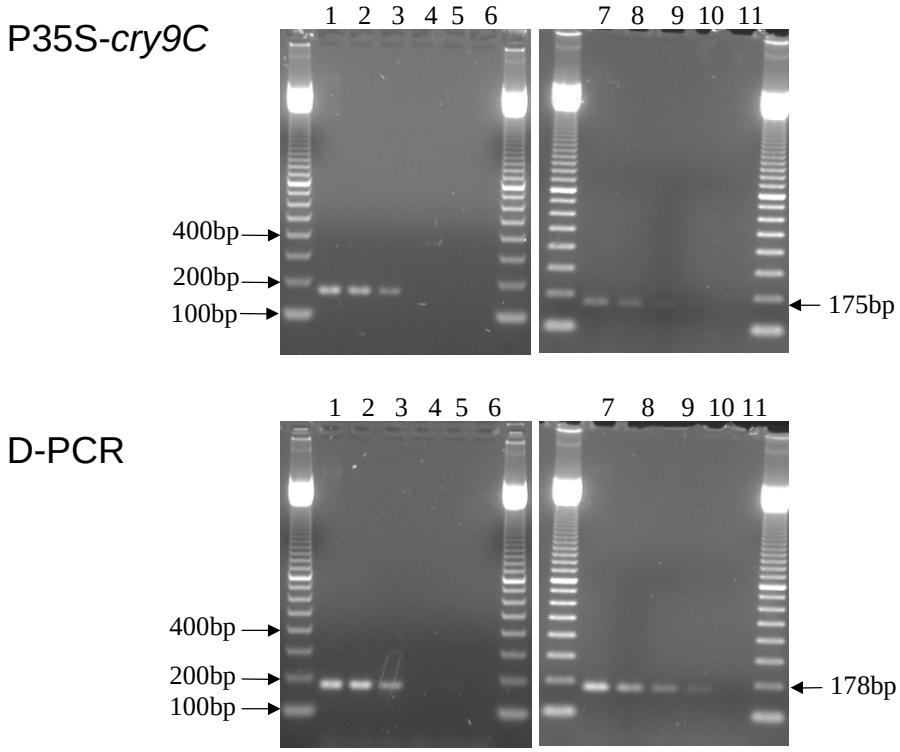

Figure 18: Agarose gel analysis - Serial dilution of genomic CHB351 DNA in isolated refined oil Control templates. Upper pannel: P35S-*cry9C* target. Lower pannel: CBH351 D-PCR target. Loading sequence of the gel:
Lanes 1: 2 µl water fortified with 10 ng genomic CBH351 DNA.
Lanes 2: 2 µl water fortified with 1 ng genomic CBH351 DNA
Lanes 3: 2 µl water fortified with 0.1 ng genomic CBH351 DNA
Lanes 4: 2 µl water fortified with 0.01 ng genomic CBH351 DNA
Lanes 5: 2 µl water fortified with 0.001 ng genomic CBH351 DNA
Lanes 6: 2 µl water
Lanes 7: 2 µl refined oil template fortified with 10 ng genomic CBH351 DNA
Lanes 8: 2 µl refined oil template fortified with 1 ng genomic CBH351 DNA
Lanes 9: 2 µl refined oil template fortified with 0.1 ng genomic CBH351 DNA
Lanes 10: 2 µl refined oil template fortified with 0.01 ng genomic CBH351 DNA
Lanes 11: 2 µl refined oil template fortified with 0.001 ng genomic CBH351 DNA
MW marker: 100 bp ladder (Amersham Pharmacia Biotech).

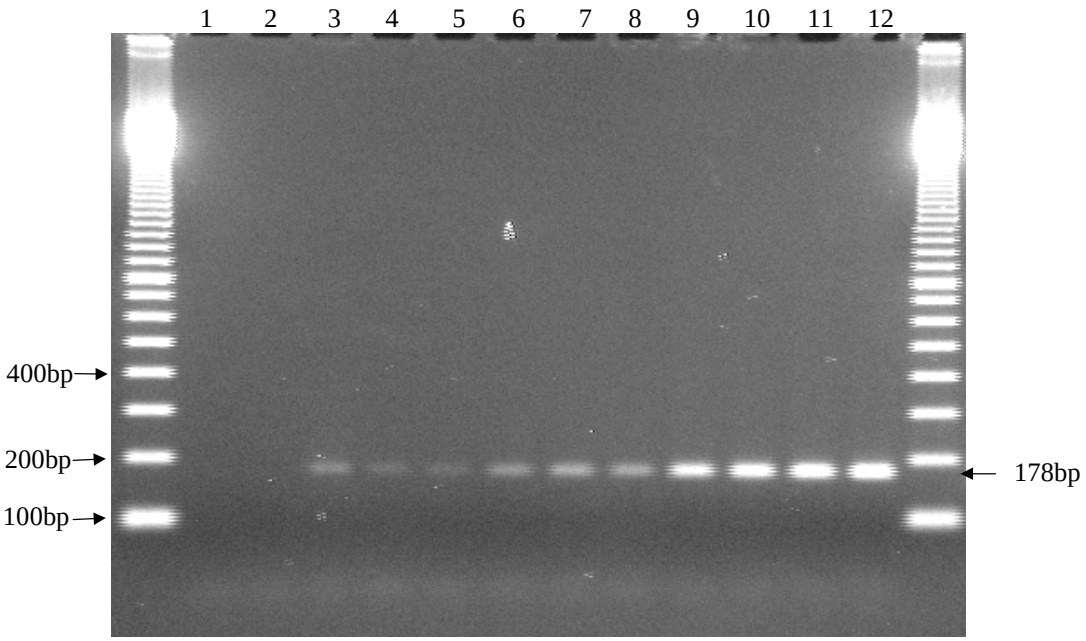

Figure 19: Agarose gel analysis - CBH351 D-PCR target in refined oil Control samples fortified with genomic CBH351.
The efficiency of the applied DNA extraction method was determined by fortification of refined oil Control samples with genomic CBH351 DNA. DNA was extracted in duplicate and subjected to PCR analysis. Loading sequence of the gel:
Lanes 1 - 2: 3 ml refined oil Control sample fortified with 0 ng genomic CBH351 DNA
Lanes 3 - 4: 3 ml refined oil Control sample fortified with 2.5 ng genomic CBH351 DNA
Lanes 5 - 6: 3 ml refined oil Control sample fortified with 5 ng genomic CBH351 DNA
Lanes 7 - 8: 3 ml refined oil Control sample fortified with 10 ng genomic CBH351 DNA
Lanes 9 - 10: 3 ml refined oil Control sample fortified with 20 ng genomic CBH351 DNA
Lanes 11 - 12: 3 ml refined oil Control sample fortified with 50 ng genomic CBH351 DNA
MW marker: 100 bp ladder (Amersham Pharmacia Biotech)

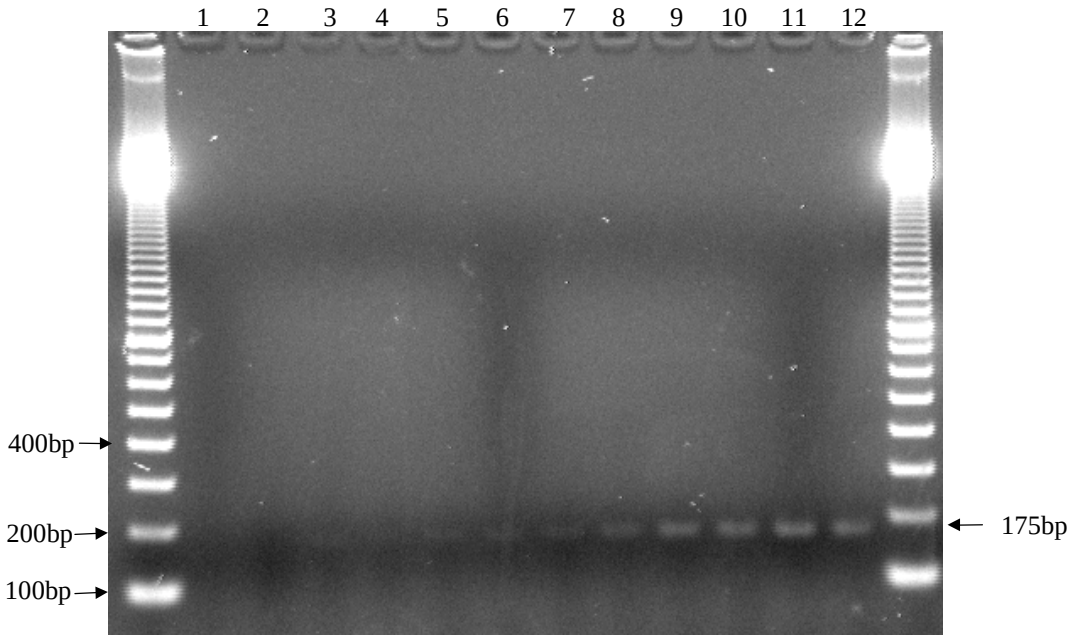

Figure 20: Agarose gel analysis - P35S-*cry9C* target in refined oil Control samples fortified with genomic CBH351.
The efficiency of the applied DNA extraction method was determined by fortification of refined oil Control samples with genomic CBH351 DNA. DNA was extracted in duplicate and subjected to PCR analysis. Loading sequence of the gel:
Lanes 1 - 2: 3 ml refined oil Control sample fortified with 0 ng genomic CBH351 DNA
Lanes 3 - 4: 3 ml refined oil Control sample fortified with 2.5 ng genomic CBH351 DNA
Lanes 5 - 6: 3 ml refined oil Control sample fortified with 5 ng genomic CBH351 DNA
Lanes 7 - 8: 3 ml refined oil Control sample fortified with 10 ng genomic CBH351 DNA
Lanes 9 - 10: 3 ml refined oil Control sample fortified with 20 ng genomic CBH351 DNA
Lanes 11 - 12: 3 ml refined oil Control sample fortified with 50 ng genomic CBH351 DNA
MW marker: 100 bp ladder (Amersham Pharmacia Biotech)

TORTILLAS (soft)

Table 11: Tortillas (soft) fraction

N(*)	BT-ID	ENDO	P35S-*Cry9C*	CBH351 D-PCR
1a	418N (Control sample)	yes	no	no
1b	418N (Control sample)	yes	no	no
2a	418N (Control sample)	yes	no	no
2b	418N (Control sample)	yes	no	no
3a	414A (StarLink™ sample)	yes	yes	yes
3b	414A (StarLink™ sample)	yes	yes	yes
4a	414A (StarLink™ sample)	yes	yes	yes
4b	414A (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 21, 22 and 23.

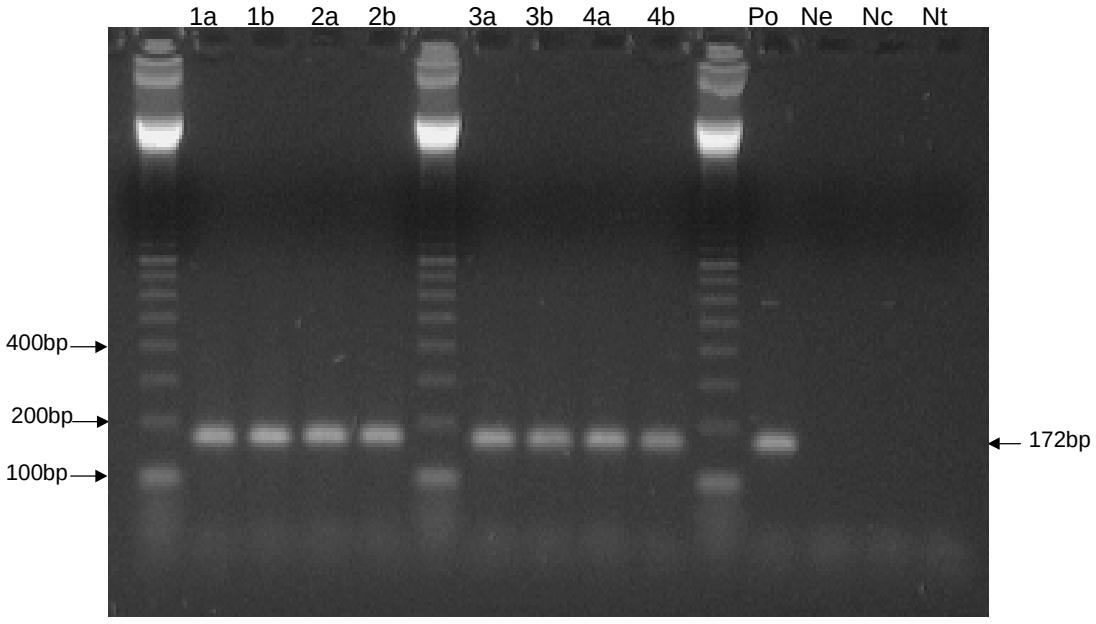

Figure 21: Agarose gel analysis - Endogenous target in tortilla (soft) Control and StarLink™ samples. Loading sequence of the gel according to Table 11.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

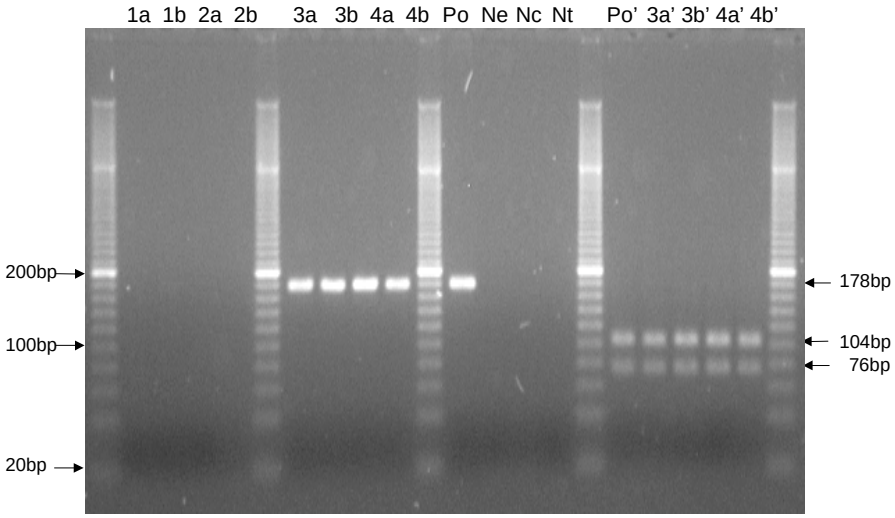

Figure 22: Agarose gel analysis - CBH351 D-PCR target in tortilla (soft) Control and StarLink™ samples. Loading sequence of the gel according to Table 11.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

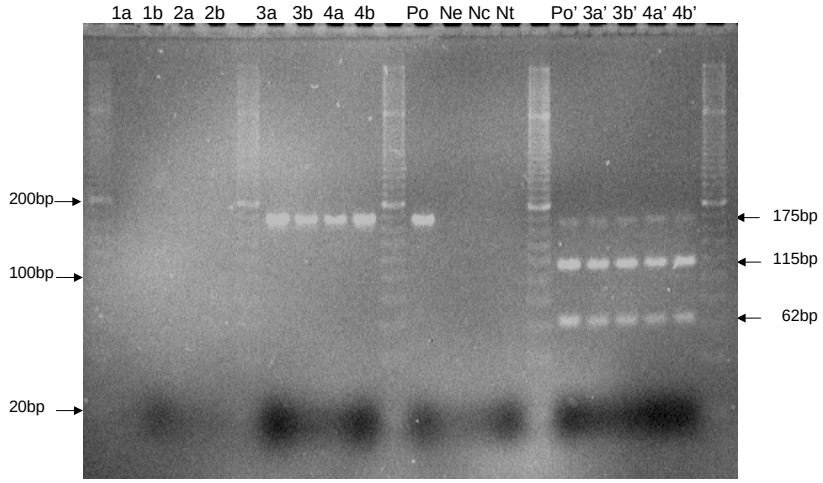

Figure 23: Agarose gel analysis - P35S-*cry9C* target in tortilla (soft) Control and StarLink™ samples. Loading sequence of the gel according to Table 11.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

TORTILLAS (fried)

Table 12: Tortillas (fried) fraction

N(*)	BT-ID	ENDO	P35S-*cry9C*	CBH351 D-PCR
1a	418M (Control sample)	yes	no	no
1b	418M (Control sample)	yes	no	no
2a	418M (Control sample)	yes	no	no
2b	418M (Control sample)	yes	no	no
3a	414B (StarLink™ sample)	yes	yes	yes
3b	414B (StarLink™ sample)	yes	yes	yes
4a	414B (StarLink™ sample)	yes	yes	yes
4b	414B (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 24, 25 and 26.

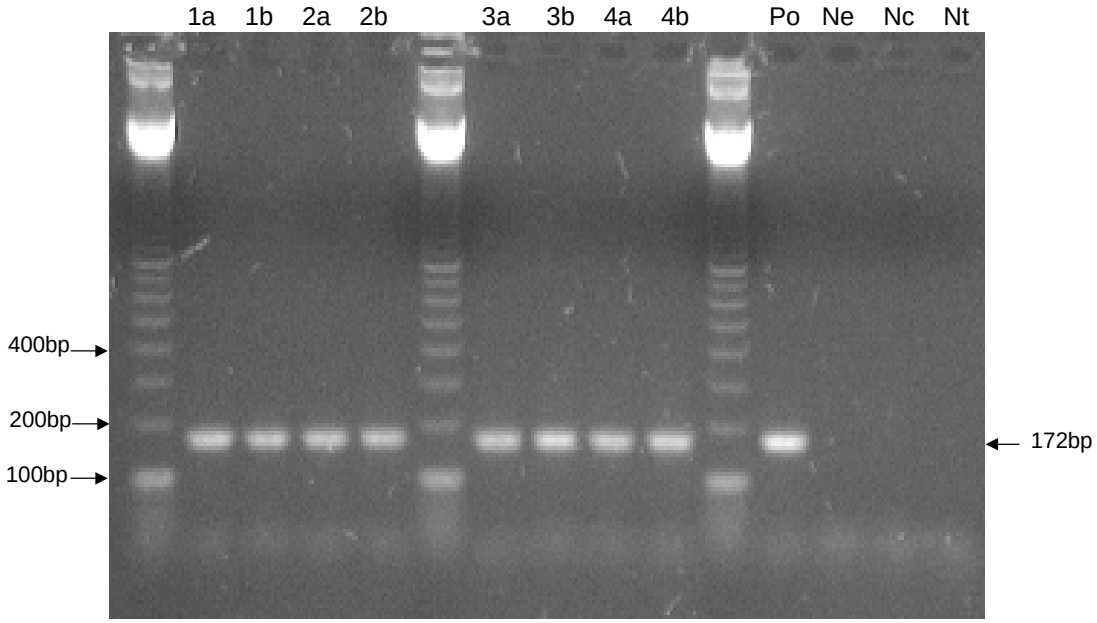

Figure 24: Agarose gel analysis - Endogenous target in tortilla (fried) Control and StarLink™ samples. Loading sequence of the gel according to Table 12.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

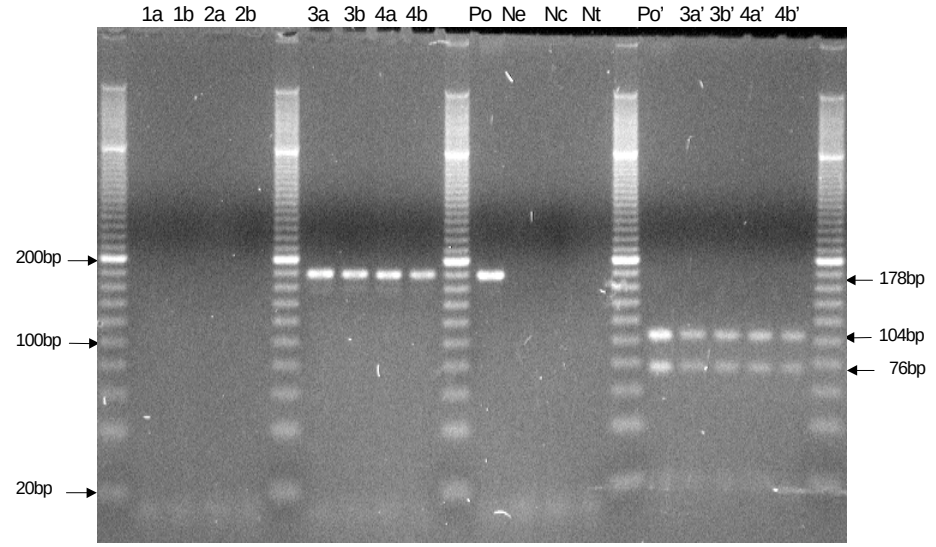

Figure 25: Agarose gel analysis - CBH351 D-PCR target in tortilla (fried) Control and StarLink™ samples. Loading sequence of the gel according to Table 12.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

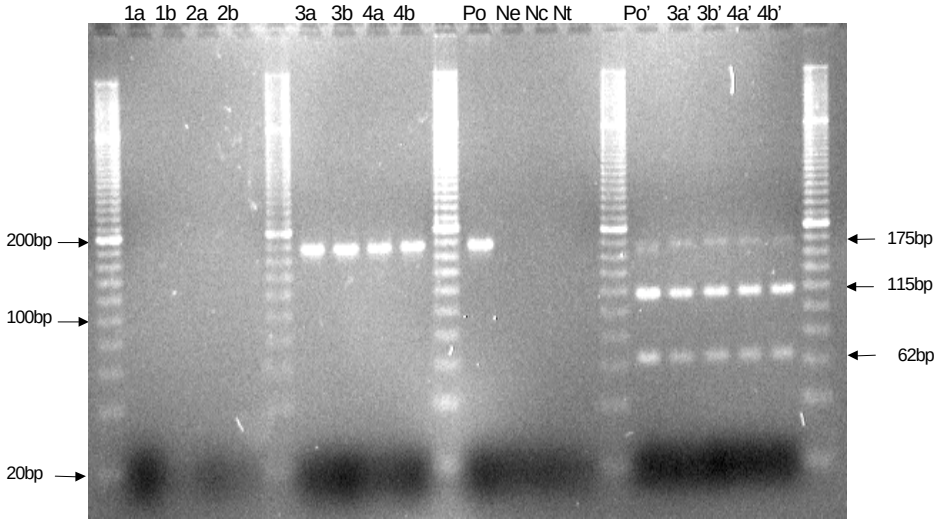

Figure 26: Agarose gel analysis - P35S-*cry9C* target in tortilla (fried) Control and StarLink™ samples. Loading sequence of the gel according to Table 12.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

PUFFED CEREALS

Table 13: Puffed Cereals fraction

N(*)	BT-ID	ENDO	P35S-*cry9C*	CBH351 D-PCR
1a	450A (Control sample)	yes	no	no
1b	450A (Control sample)	yes	no	no
2a	450A (Control sample)	yes	no	no
2b	450A (Control sample)	yes	no	no
3a	450C (StarLink™ sample)	yes	yes	yes
3b	450C (StarLink™ sample)	yes	yes	yes
4a	450C (StarLink™ sample)	yes	yes	yes
4b	450C (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 27, 28 and 29.

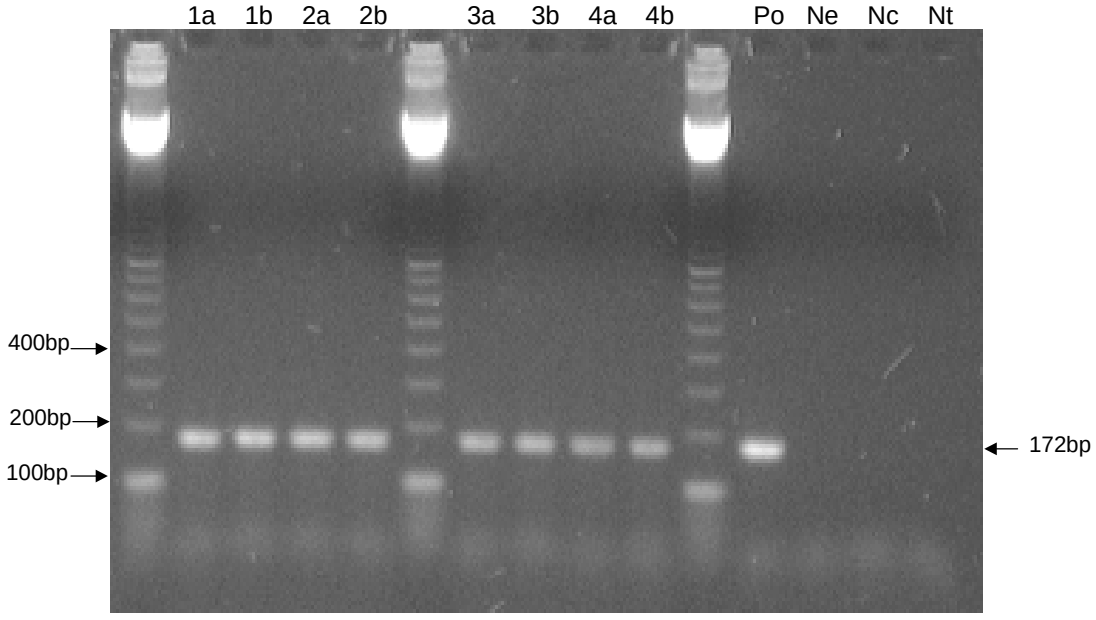

Figure 27: Agarose gel analysis - Endogenous target in puffed cereal Control and StarLink™ samples. Loading sequence of the gel according to Table 13.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

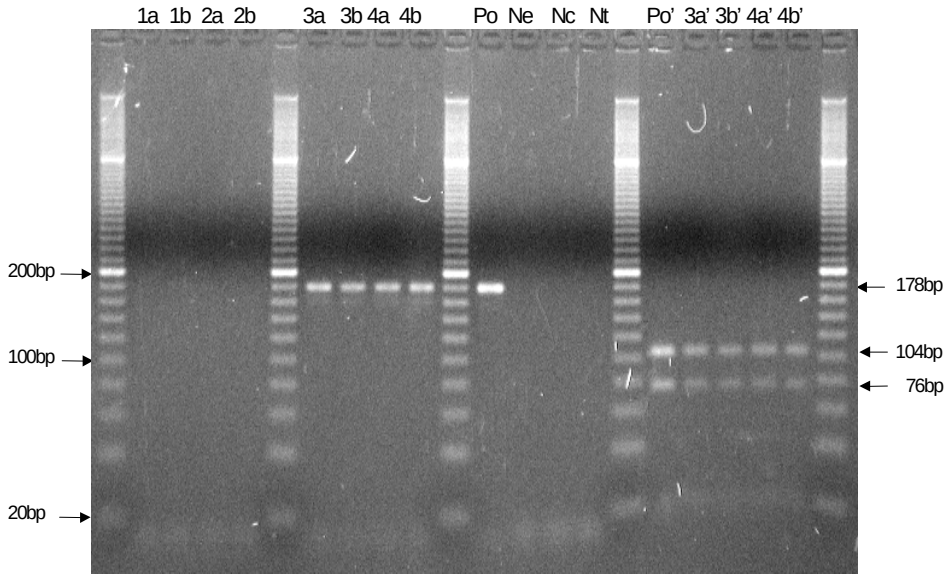

Figure 28: Agarose gel analysis - CBH351 D-PCR target in puffed cereal Control and StarLink™ samples. Loading sequence of the gel according to Table 13.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

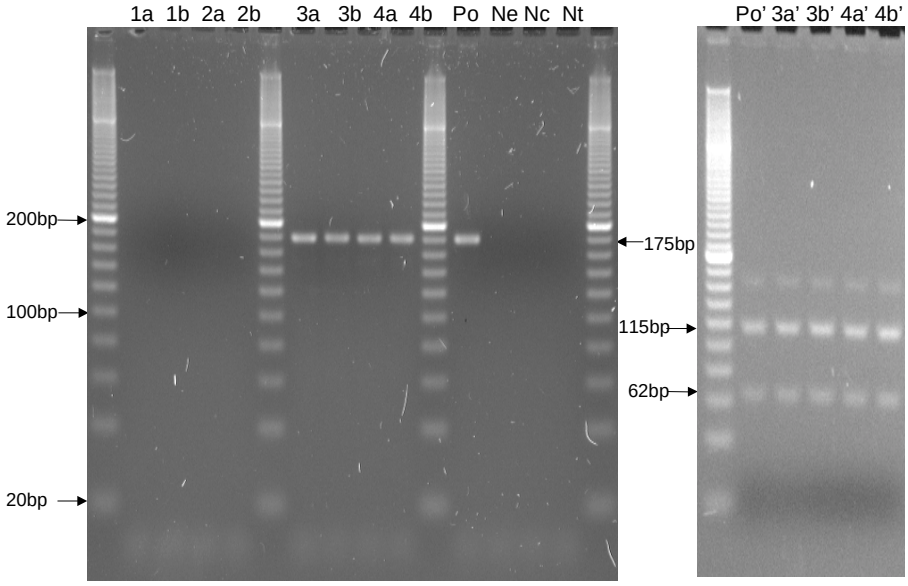

Figure 29: Agarose gel analysis - P35S-*cry9C* target in puffed cereal Control and StarLink™ samples. Loading sequence of the gel according to Table 13.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

CORN PUFFS

Table 14: Corn Puffs fraction

N(*)	BT-ID	ENDO	P35S-*Cry9C*	CBH351 D-PCR
1a	452A (Control sample)	yes	no	no
1b	452A (Control sample)	yes	no	no
2a	452A (Control sample)	yes	no	no
2b	452A (Control sample)	yes	no	no
3a	452B (StarLink™ sample)	yes	yes	yes
3b	452B (StarLink™ sample)	yes	yes	yes
4a	452B (StarLink™ sample)	yes	yes	yes
4b	452B (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 30, 31 and 32.

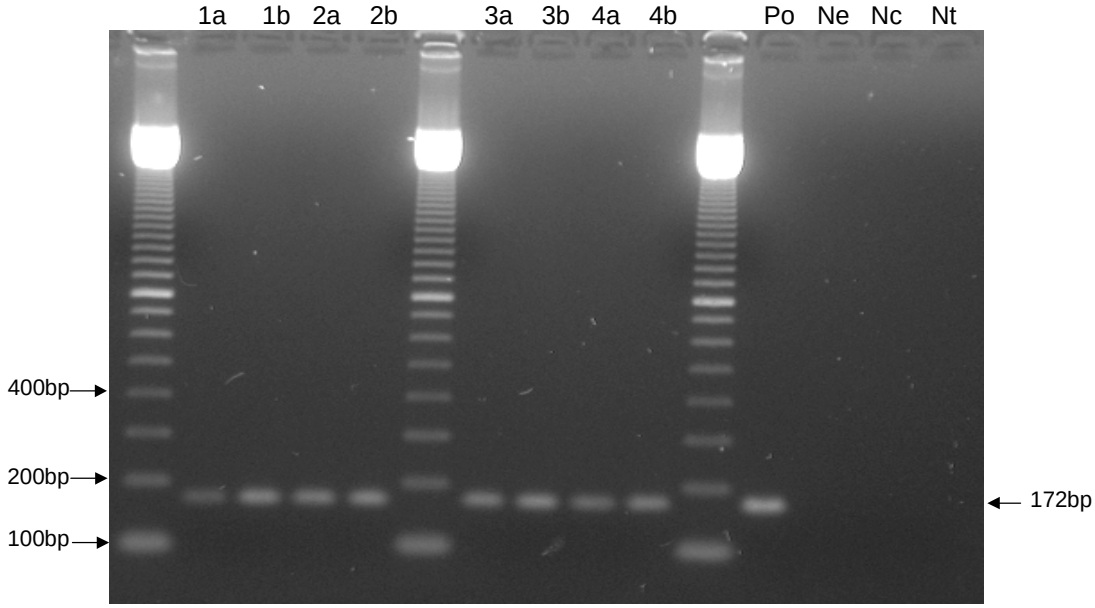

Figure 30: Agarose gel analysis - Endogenous target in corn puffs Control and StarLink™ samples. Loading sequence of the gel according to Table 14.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

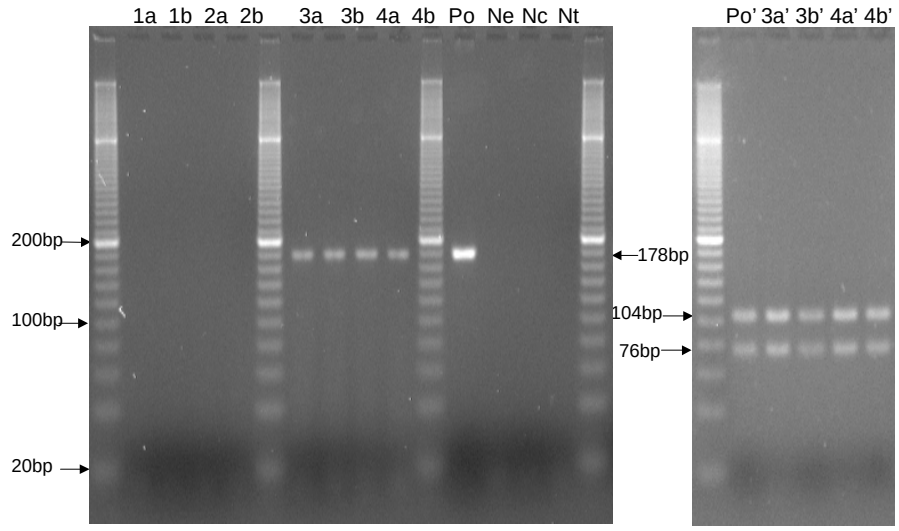

Figure 31: Agarose gel analysis - CBH351 D-PCR target in corn puffs Control and StarLink™ samples. Loading sequence of the gel according to Table 14.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

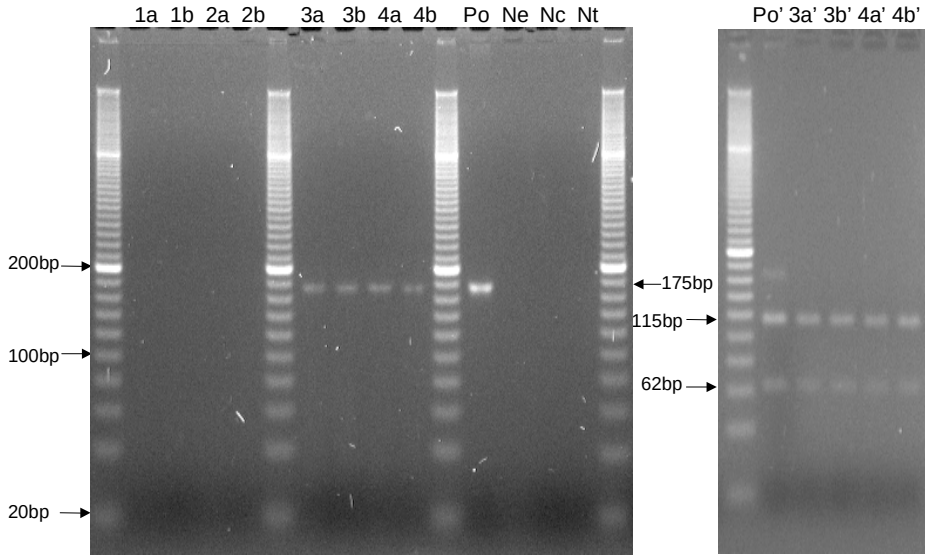

Figure 32: Agarose gel analysis - P35S-*cry9C* target in corn puffs Control and StarLink™ samples. Loading sequence of the gel according to Table 14.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

CORN FLAKES

Table 15: Corn Flakes fraction

N(*)	BT-ID	ENDO	P35S-*Cry9C*	CBH351 D-PCR
1a	453A (Control sample)	yes	no	no
1b	453A (Control sample)	yes	no	no
2a	453A (Control sample)	yes	no	no
2b	453A (Control sample)	yes	no	no
3a	453B (StarLink™ sample)	yes	yes	yes
3b	453B (StarLink™ sample)	yes	yes	yes
4a	453B (StarLink™ sample)	yes	yes	yes
4b	453B (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 33, 34 and 35.

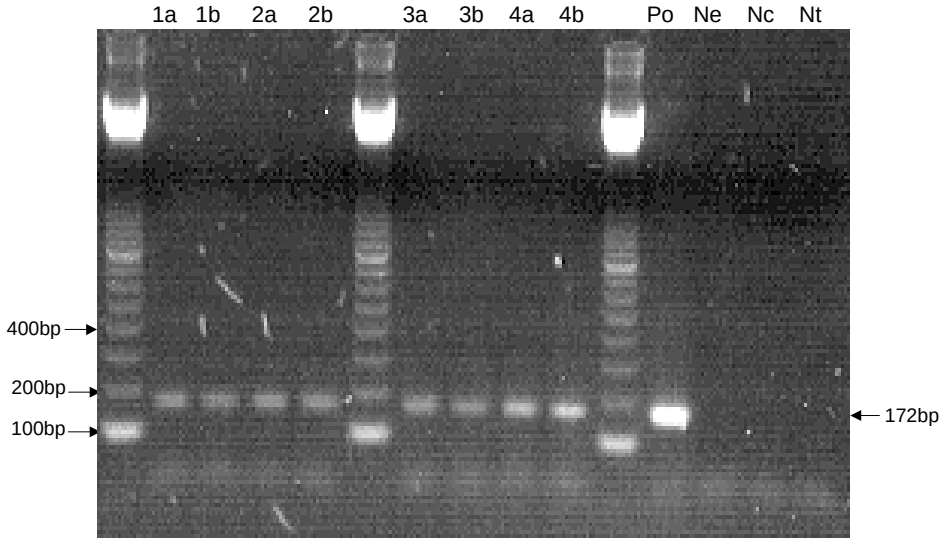

Figure 33: Agarose gel analysis - Endogenous target in corn flakes Control and StarLink™ samples. Loading sequence of the gel according to Table 15.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

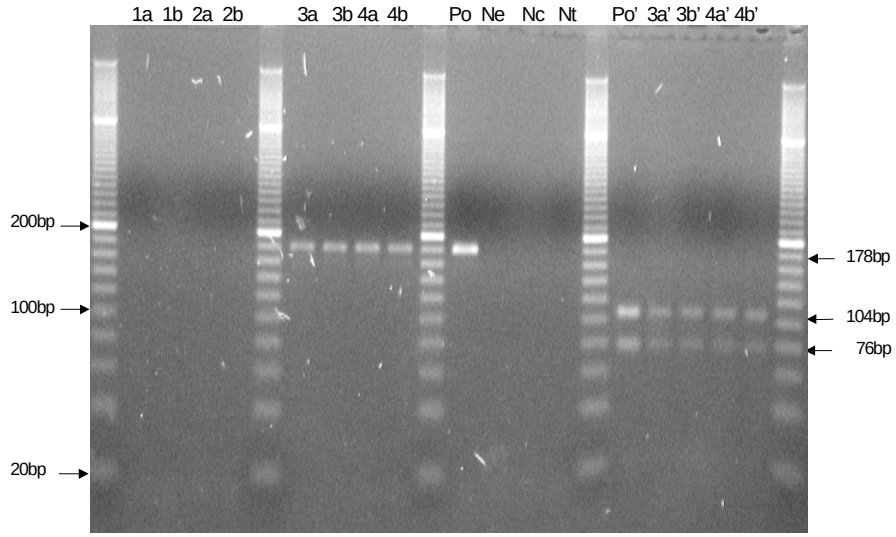

Figure 34: Agarose gel analysis - CBH351 D-PCR target in corn flakes Control and StarLink™ samples. Loading sequence of the gel according to Table 15.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

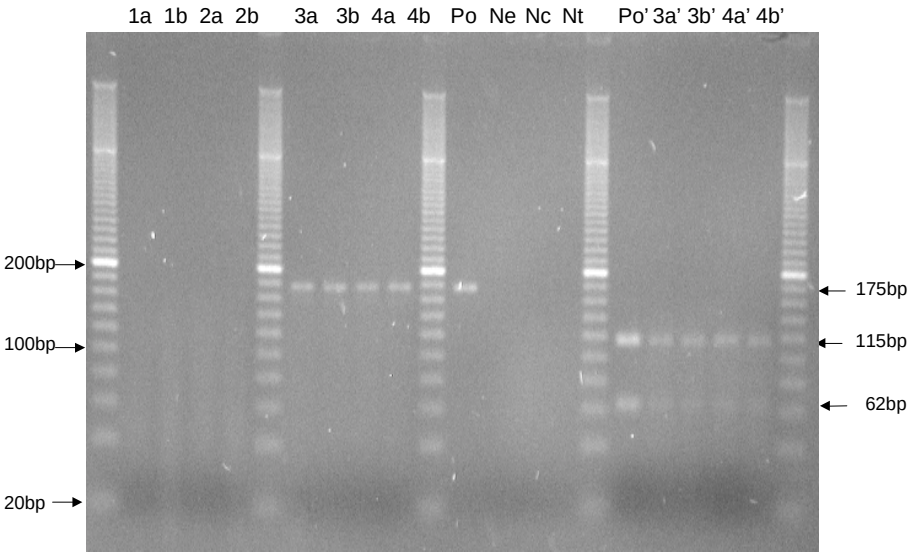

Figure 35: Agarose gel analysis - P35S-*Cry9C* target in corn flakes Control and StarLink™ samples. Loading sequence of the gel according to Table 15.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

CORN MUFFINS

Table 16: Corn Muffin fraction

N(*)	BT-ID	ENDO	P35S-*Cry9C*	CBH351 D-PCR
1a	456E (Control sample)	yes	no	no
1b	456E (Control sample)	yes	no	no
2a	456E (Control sample)	yes	no	no
2b	456E (Control sample)	yes	no	no
3a	456H (StarLink™ sample)	yes	yes	yes
3b	456H (StarLink™ sample)	yes	yes	yes
4a	456H (StarLink™ sample)	yes	yes	yes
4b	456H (StarLink™ sample)	yes	yes	yes
5a	456F (Control sample)	yes	no	no
5b	456F (Control sample)	yes	no	no
6a	456F (Control sample)	yes	no	no
6b	456F (Control sample)	yes	no	no
7a	456J (StarLink™ sample)	yes	yes	yes
7b	456J (StarLink™ sample)	yes	yes	yes
8a	456J (StarLink™ sample)	yes	yes	yes
8b	456J (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 36, 37 and 38.

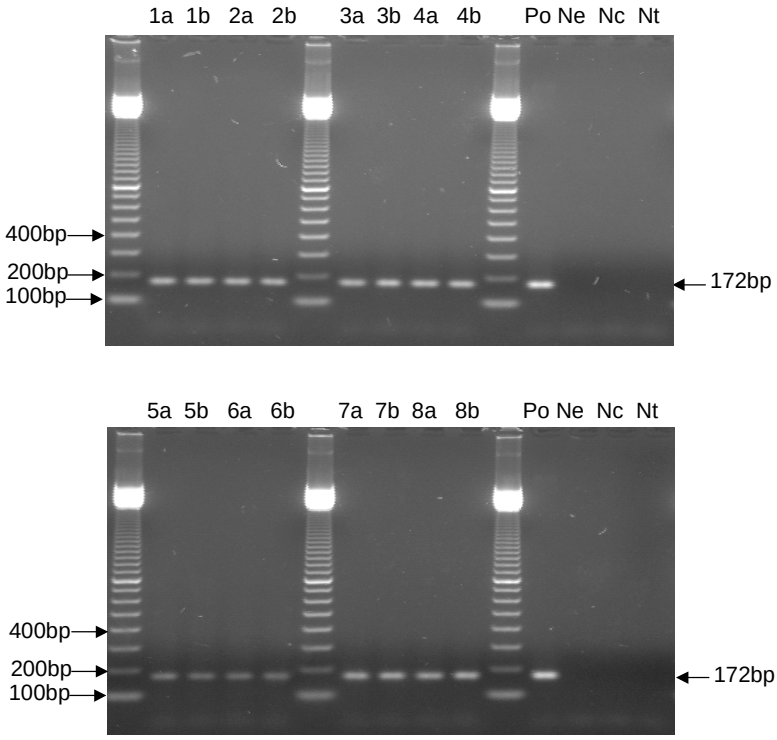

Figure 36: Agarose gel analysis - Endogenous target in corn muffin Control and StarLink™ samples. Loading sequence of the gel according to Table 16.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

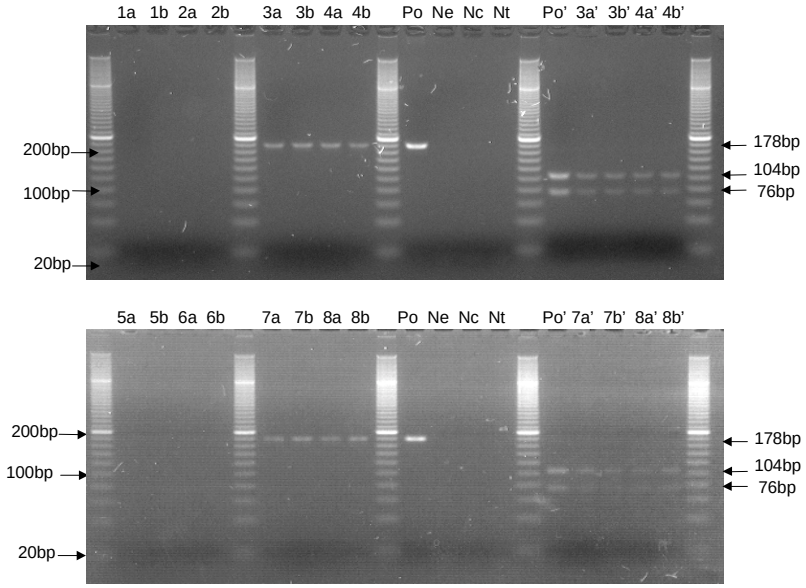

Figure 37: Agarose gel analysis - CBH351 D-PCR target in corn muffin Control and StarLink™ samples. Loading sequence of the gels according to Table 16.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b', 7a', 7b', 8a' and 8b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a, 4b, 7a, 7b, 8a and 8b.

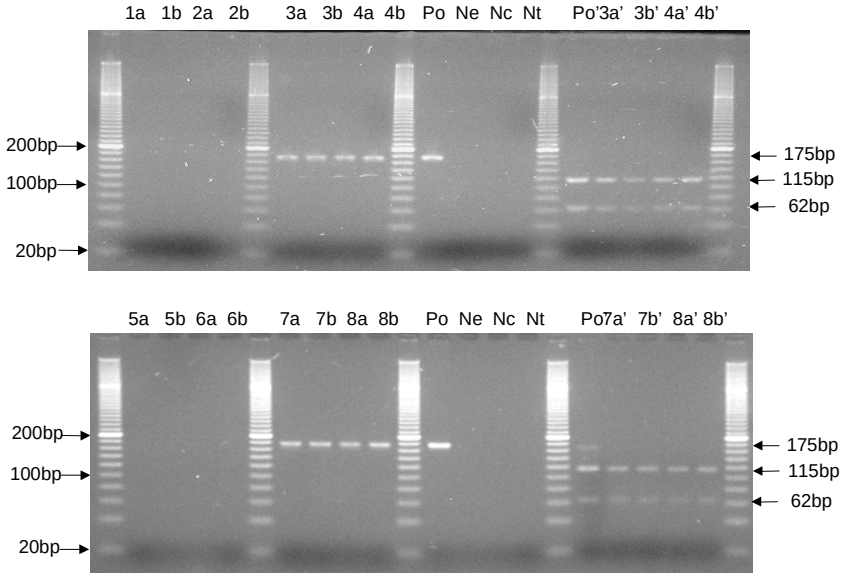

Figure 38: Agarose gel analysis - P35S-*cry9C* target in corn muffin Control and StarLink™ samples. Loading sequence of the gels according to Table 16.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b', 7a', 7b', 8a' and 8b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a, 4b, 7a, 7b, 8a and 8b.

CORN BREAD

Table 17: Corn Bread fraction

N(*)	BT-ID	ENDO	P35S-*Cry9C*	CBH351 D-PCR
1a	456K (Control sample)	yes	no	no
1b	456K (Control sample)	yes	no	no
2a	456K (Control sample)	yes	no	no
2b	456K (Control sample)	yes	no	no
3a	456N (StarLink™ sample)	yes	yes	yes
3b	456N (StarLink™ sample)	yes	yes	yes
4a	456N (StarLink™ sample)	yes	yes	yes
4b	456N (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 39, 40 and 41.

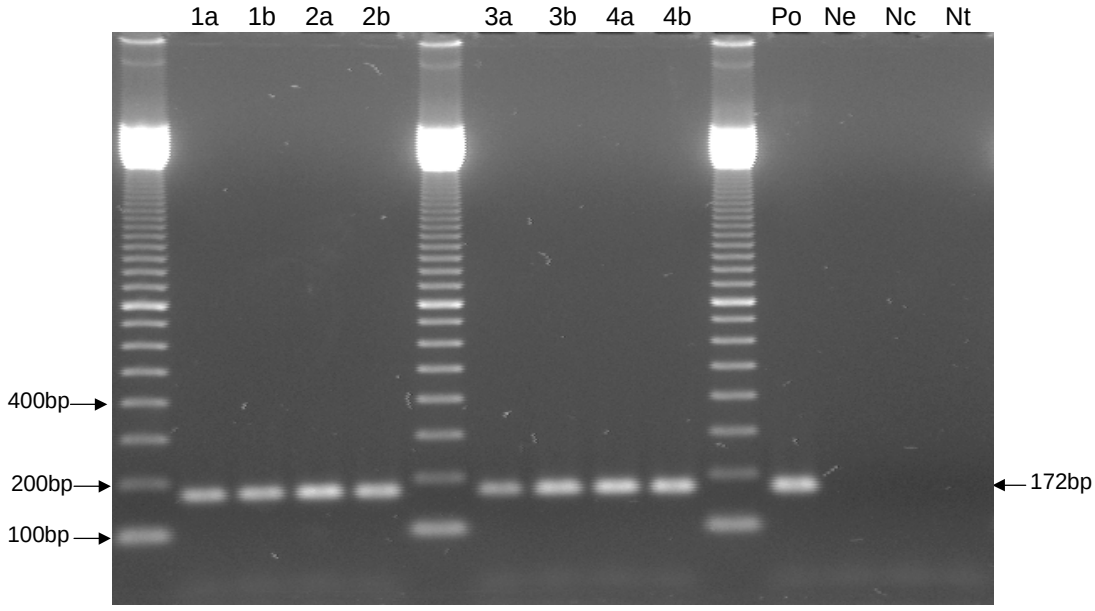

Figure 39: Agarose gel analysis - Endogenous target in corn bread Control and StarLink™ samples. Loading sequence of the gel according to Table 17.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

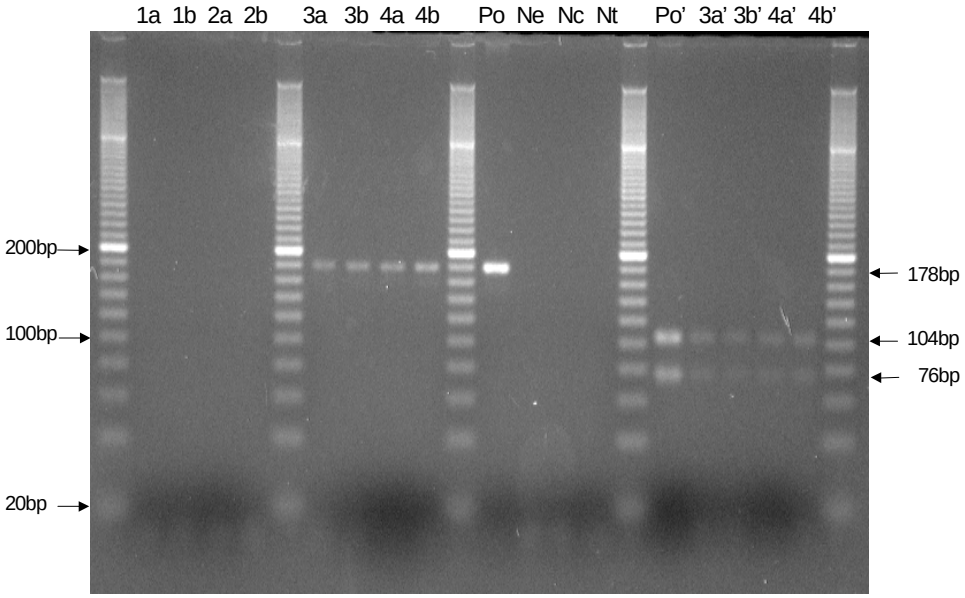

Figure 40: Agarose gel analysis - CBH351 D-PCR target in corn bread Control and StarLink™ samples. Loading sequence of the gel according to Table 17.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

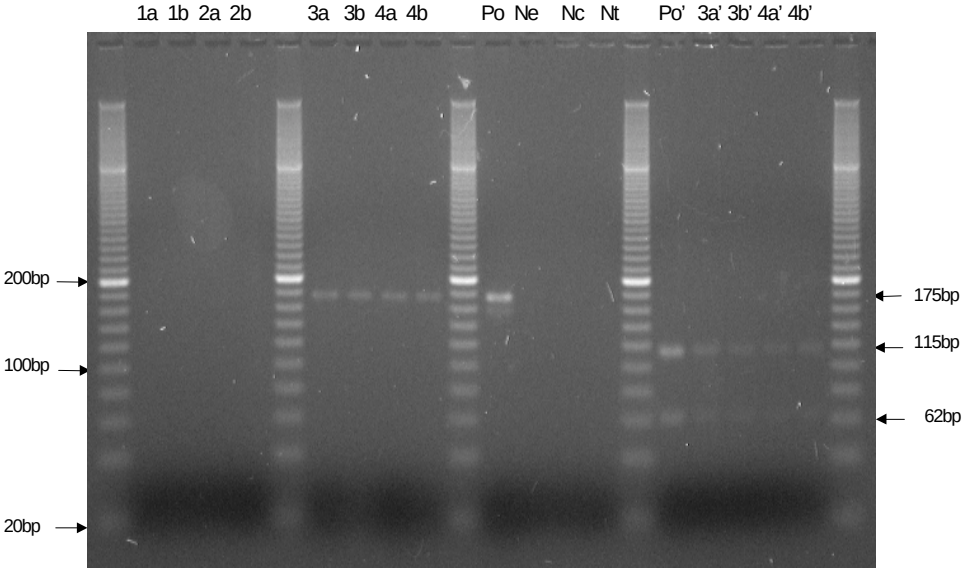

Figure 41: Agarose gel analysis - P35S-*cry9C* target in corn bread Control and StarLink™ samples. Loading sequence of the gel according to Table 17.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

POLENTA

Table 18: Polenta fraction

N(*)	BT-ID	ENDO	P35S-*Cry9C*	CBH351 D-PCR
1a	456B (Control sample)	yes	no	no
1b	456B (Control sample)	yes	no	no
2a	456B (Control sample)	yes	no	no
2b	456B (Control sample)	yes	no	no
3a	456D (StarLink™ sample)	yes	yes	yes
3b	456D (StarLink™ sample)	yes	yes	yes
4a	456D (StarLink™ sample)	yes	yes	yes
4b	456D (StarLink™ sample)	yes	yes	yes

(*) Loading sequence of Figures 42, 43 and 44.

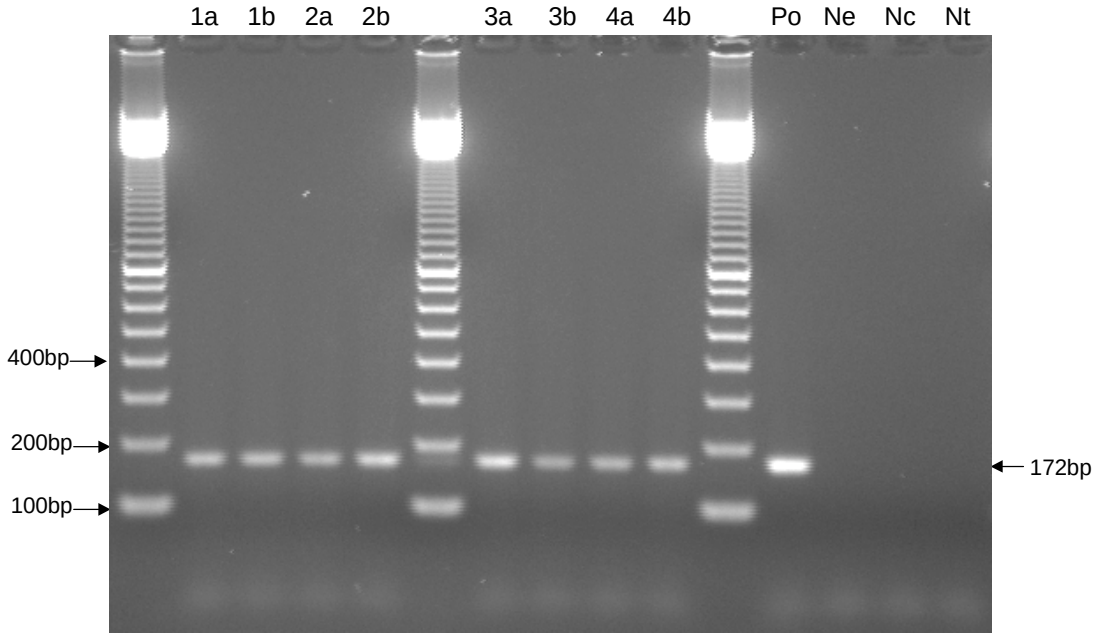

Figure 42: Agarose gel analysis - Endogenous target in polenta Control and StarLink™ samples. Loading sequence of the gel according to Table 18.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control.

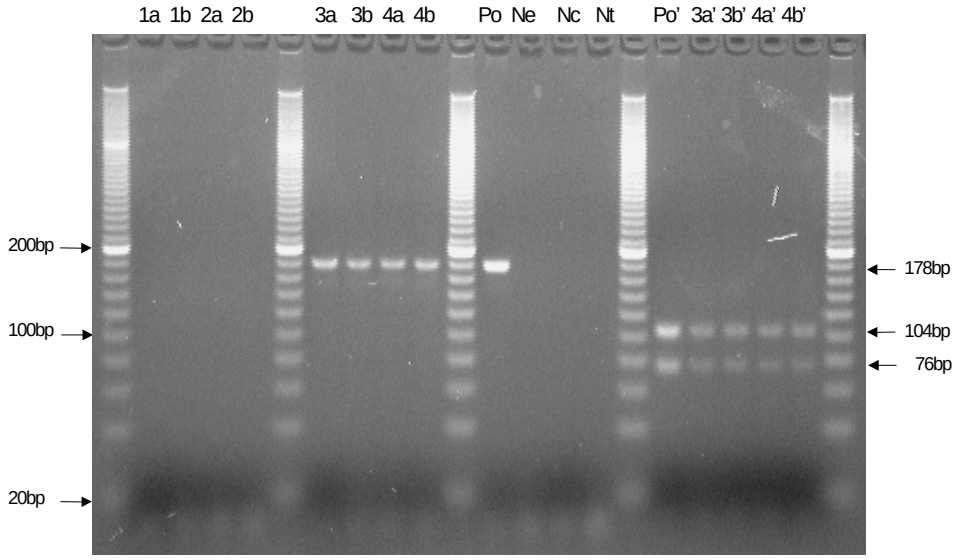

Figure 43: Agarose gel analysis - CBH351 D-PCR target in polenta Control and StarLink™ samples. Loading sequence of the gel according to Table 18.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *HhaI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.

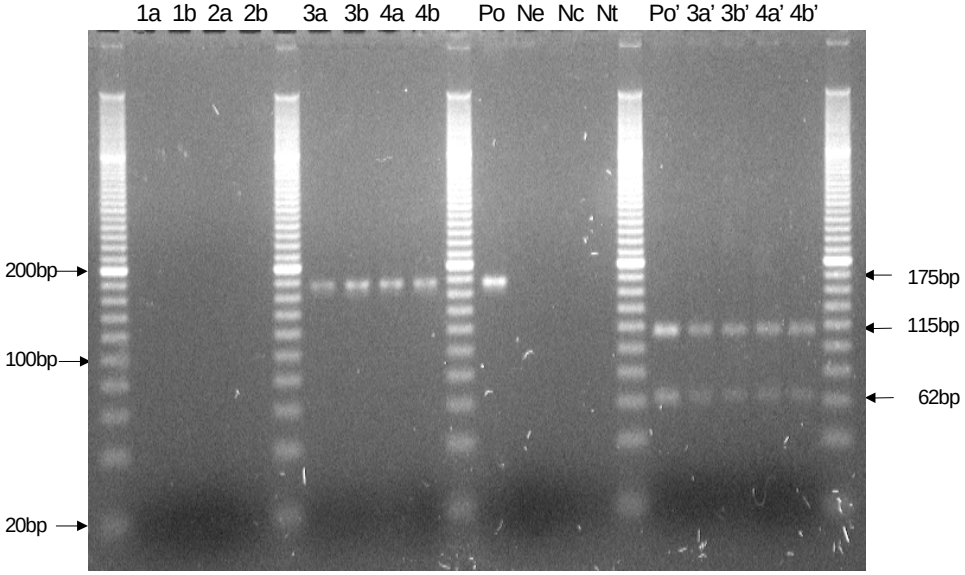

Figure 44: Agarose gel analysis - P35S-*cry9C* target in polenta Control and StarLink™ samples. Loading sequence of the gel according to Table 18.
Po = Positive control. Ne = Negative control. Nc = No cross-contamination control. Nt = No template control. Lanes Po', 3a', 3b', 4a', 4b': *MseI* restriction enzyme analysis on the PCR products of respectively Po, 3a, 3b, 4a and 4b.